AWARD NUMBER: W81XWH-15-1-0515

TITLE: **Neurovascular and Autonomic Dysfunction Associated with Gulf War Illness Pain**

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REPORT DATE: October 2017

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command

Fort Detrick, Maryland, 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
xxxxxxxx Oct 2017	Annual	30 Sep 2016 - 29 Sep 2017

4. TITLE AND SUBTITLE

Neurovascular and Autonomic Dysfunction Associated with Gulf War Illness Pain

Neurovascular and Autonomic Dysfunction Associated with Gulf War Illness Pain

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-15-1-0515

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Florida
207 Grinter Hall
Gainesville, Fl
32611-5500

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Soldiers returning from the 1991 Persian Gulf War suffered from a variety of cognitive, motor, sensory and autonomic symptoms. We have shown that a 4 week exposure to DEET, chlorpyrifos, permethrin and PB produced pain-like signs and chronic vasodilation in rats that persisted up to 24 weeks after exposure. The onset and extent of these signs were increased by inclusion of DEET in the exposure protocol. The appearance of pain signs were dependent on the inclusion of anticholinesterases (chlorpyrifos or PB). Exclusion of either chlorpyrifos or PB prevented the development of behavioral deficits and autonomic maladaptations (Flunker et al., 2017). We had previously demonstrated that nociceptors from rats exhibiting pain behaviors exhibited defects in the activity of nociceptor ion channels (K_v7: Nutter et al., 2015; $Na_v1.9$: Nutter and Cooper, 2014). We have now replicated the effect on $Na_v1.9$ and demonstrated that it was up-regulated up to 16 weeks after exposures had ended. Using the demonstrated capacity of PB to control the appearance of pain signs we have further shown that deficits in K_v7 did not vary with pain signs exhibited in PB exposed or non-exposed rats (Flunker et al., 2017); moreover, they did not persist past 12 weeks post-exposure. Therefore, shifts in K_v7 activity were not fundamental to the development or maintenance of pain in our model. Studies examining the capacity of FDA approved drugs to manage pain signs in exposed rats were partially successful. During a 4 week treatment with the K_v7 opener Retigabine (1200 mg/kg/day), rat pain signs were significantly improved; however, pain signs rapidly reappeared once treatments ceased. Given the function of K_v7, the capacity of a K_v7 active drug to improve pain signs, even though K_v7 ion channels were not directly involved in the pathophysiology of GWI pain, was not unexpected nor implausible. Yet it is not likely that targeting this ion channel would constitute a broad or permanent cure for symptoms of GWI. K_v7 openers could be effective palliatives for GWI pain.

15. SUBJECT TERMS
Pain, pesticides, pyridostigmine bromide, Retigabine, DEET, chlorpyrifos, Gulf War Illness

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
Unclassified	Unclassified	Unclassified	Unclassified	116	19b. TELEPHONE NUMBER *(include area code)*

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1. Introduction

Pain and autonomic dysfunction are common symptoms associated with Gulf War Illness. Thousands of soldiers returning from the Persian Gulf War developed unusual complexes of headache, joint, muscle and abdominal pains (GWI; Haley Syndrome 3; Haley and Kurt, 1997; Blanchard et al., 2006; Stimpson et al., 2006; Thomas et al., 2006; Haley et al., 2013). Chronic deep tissue pain was often accompanied by dizziness, night sweats, diarrhea and a variety of other signs of autonomic dysfunction that were also manifested with the cognitive and motor symptom complexes of GWI (Haley Syndromes 1 and 2; Haley and Kurt, 1997; Haley et al., 2013). To investigate the pathophysiology of this disorder, our laboratory developed a rat model of GWI pain. Following a series of studies that utilized a variety of exposure protocols, we demonstrated that pain-like behaviors emerged 12 weeks after an 8 week exposure to a combination of permethrin, chlorpyrifos and pyridostigmine bromide. These behavioral signs were associated with decreased activity of nociceptor K_V7 and maladapted reactivity to muscarinic acetylcholine receptors (mAChR; Nutter et al., 2015; Cooper et al., 2016).

It had been reported that application of high concentrations of, the insect repellant, DEET was uniquely associated the pain symptoms of GWI (Haley and Kurt, 1997). In the project covered by this report, we examined the contribution of DEET to the development and persistence of post-exposure pain behaviors. Last year we reported that adding DEET to the exposure protocol (permethrin, chlorpyrifos and PB) accelerated and prolonged the pain signs that developed after a 4 week exposure. No specific interactions were found between acute exposure to DEET and those chronic molecular maladaptations associated with prolonged to 4 GW chemicals (Flunker et al., 2017). At present, it is more likely that DEET indirectly amplifies the physiological impact of the anticholinesterases on their molecular targets by interfering with hepatic catabolism of

anticholinesterases. Despite the impact of DEET on the development of pain signs, it was clear from our studies that the appearance of these signs was completely dependent on the anticholinesterases (PB and chlorpyrifos). In the second year of the project we examined the persistence of molecular maladaptations that had been previously associated with the 3 agent exposure (K_v7, $Na_v1.9$), and determined whether treatment with FDA approved medications could reverse pain behaviors. A special emphasis was placed on the evaluation of autonomic dysfunction that can accompany pain symptoms in veterans with GWI.

2. **Keywords:** pain, autonomic, nociceptor, blood flow, pesticides, pyridostigmine bromide, DEET, Gulf War Illness

3. Accomplishments

The SOW is presented in the Appendix (page 41). The year 2 objectives are outlined below:

Objectives Year 2:

1) Characterize molecular changes that occur in nociceptors following exposure to a DEET augmented exposure protocol pain at 16 weeks post-exposure.
2) Characterize autonomic dysfunctions that occur following exposure to a DEET augmented exposure protocol pain at 16 weeks post-exposure.
3) Characterize alterations of vascular nociceptor function, in vivo, in GW chemical exposed rats.
4) Determine whether FDA approved drugs can be repurposed to reverse pain behaviors of rats in our model of GWI pain.

TASK 1.1: Optimize the Chemical Exposure Protocol

Timeline: Months 1-5

We determined the contributions of DEET, chlorpyrifos, permethrin and pyridostigmine bromide to the development and persistence of pain behaviors. These data were presented in the year 1 report.

Specific Aim 1. Reversing Signs of GWI Pain Behaviors Maintained by Vascular Nociceptors

TASK 1.2: Targeting Maladapted Ion Channel Proteins with Systemic Treatments

TASK 1.3: Targeting Maladapted Ion Channel Proteins with Multiple Systemic Treatments

We exposed groups of rats to the optimized protocol (DEET, chlorpyrifos, PB, permethrin) in order to determine whether drug treatments that targeted previously demonstrated molecular maladaptations could acutely or permanently reverse pain signs that persisted following the exposure to GW chemicals. The optimized protocol consisted of exposure to four GWI chemicals for 4 weeks (permethrin 2.6 mg/kg, chlorpyrifos 120 mg/kg, PB 13 mg/kg, DEET, 400 mg/kg; 50% in ETOH). We had demonstrated, in year 1, that the DEET augmented exposure produced rapid, robust and persistent pain signs that were suitable for drug treatment studies. Based upon our findings in TASK 2, the molecular targets for treatment drugs were K_v7 and $Na_v1.9$. We also had a strong interest in targeting CGRP (calcitonin gene related peptide) receptors, because activity in vascular nociceptors would release CGRP and result in vasodilation and certain PNS and CNS pro-inflammatory consequences associated with the release of CGRP (Li et al., 2008; De Corato et al., 2011; Malon et al., 2011).

We had previously shown that K_v7 was down regulated following exposure to either 3 GW chemicals (permethrin, chlorpyrifos and PB; 8 week exposure; Nutter et al., 2015) or 4 GW chemicals (DEET, permethrin, chlorpyrifos and PB; 4 week exposure; year 1 report; Flunker et al., 2017). Down regulation of this ion channel increases nociceptor excitability and could contribute to a chronic pain condition (Brown and Passmore, 2009).

We used the established and clinically effective K_v7 opener, Retigabine, in order to examine whether rat pain behaviors could be temporarily or permanently reversed. K_v7 openers have been used to treat some forms of chronic pain with success (Flupirtine; Devulder et al., 2010). In the studies below, we examined whether a 4 week treatment with Retigabine could acutely and/or permanently reverse the pain like symptoms that developed in our GWI model.

Retigabine Treatments Reverse Pain-Like Behaviors

Rats were prepared for behavioral studies (see Appendix, Methods, p 44). GRP A2 was composed of rats that were exposed, for 4 weeks, to DEET, permethrin, chlorpyrifos and PB. GRP C2 rats were exposed only to the corresponding vehicles. Ambulation and resting behaviors were assessed once per week.

Retigabine treatments were initiated at 9WP (9 weeks post exposure) in half of the GRP A2 rats. These treated animals were designated as GRP R. Retigabine treatment continued daily for 4 weeks (9-12WP; 1200 mg/kg/day in DMSO; oral gavage). Half of GRP A2 and all GRP C2 rats

received DMSO during the treatment phase. Behavior testing occurred daily within 2-3 hours of gavage. In order to determine the persistence of any significant Retigabine related recovery, testing continued for another month after the treatments ended (13-16WP).

Rats of GRP A2 developed pain-like behaviors manifested as a significant decrease in ambulation. The onset of effects were delayed in a manner consistent with most GW veterans (~75%; Kroenke et al., 1998). Movement distance and average rate of movement were significantly decreased at 9-12WP following the 4 week exposure to GW chemicals (figure 1A and B; $p<.01$ and $p<.002$ respectively). Movement rate was also decreased at 16WP.

Treatment with Retigabine (GRP R) produced mixed, but generally good outcomes. Retigabine rescued the exposure-induced reduction in average rate. Average movement rate of GRP R rats was significantly increased relative to vehicle treated GRP A ($p<.006$) and was not significantly depressed relative to vehicle treated (GRP C2) animals (figure 1B). These outcomes are consistent with a reduction in chronic pain behaviors. Over the years in which we have used this GWI model, rate of movement has been the most consistent measure of persistent and delayed deficits that appear following GW chemical exposure (Nutter et al., 2015; Flunker et al., 2017; see also Annual Report Year 1).

Movement distance score outcomes were less definitive. Although Retigabine treated animals 'improved' to the extent that their scores were not significantly decreased relative to GRP C2 ($p<.10$), trends were still strongly towards reduced movement distance. Moreover, Retigabine failed to significantly improve movement distance scores relative to GRP A2 rats (figure 1A).

Resting scores were not significantly affected by GW chemical exposure ($p<.12$). This is consistent with our prior published reports (Flunker et al., 2017). Retigabine paradoxically

increased resting scores, suggesting some non-specific effects (GRP R vs GRP C2; figure 2C; $p<.002$). Resting scores returned to normal levels after Retigabine treatments ended. These non-specific effects of Retigabine are not likely to have falsely impacted rate and movement scores as those manifested increased activity with Retigabine treatment.

Although Retigabine significantly improved ambulation scores during the treatment phase, once treatment with Retigabine ceased, rate of movement and distance of movement measures indicated a return of pain-influenced movements (Figure 1B; 13-16WP; Post-Treatment). That is, the treatment was successful during administration of Retigabine, but once treatment ceased, pain signs returned. Importantly, GRP A2 also continued to exhibit diminished ambulation rate relative to GRP C2 at 13-16WP ($p<.002$; Post-Treatment; figure 2B). Therefore, Retigabine treatment produced a partial but transient rescue of pain-like ambulation behaviors associated with exposure to GW chemicals.

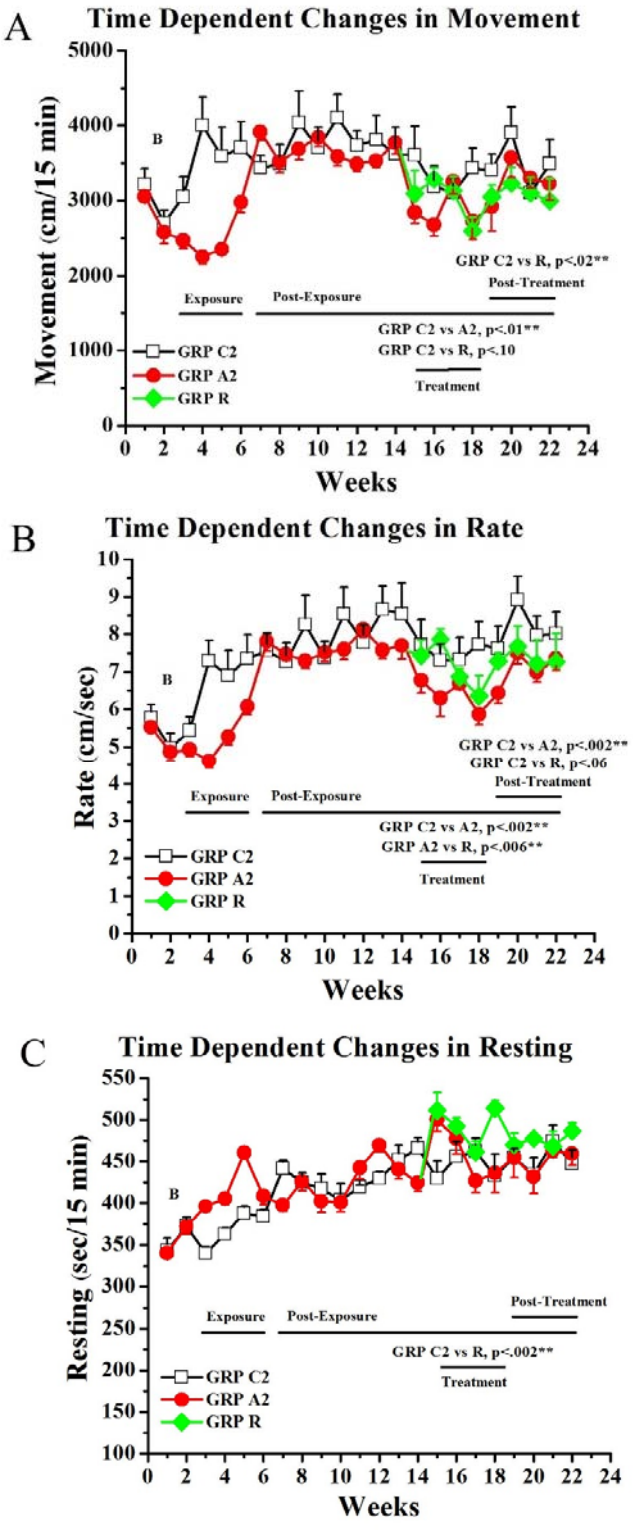

Figure 1. Treatment with Retigabine Produced a Transient Reduction of Pain-Like Behaviors. **A)** Exposure to all 4 GW agents (GRP A2) significantly decreased movement distance scores at 9-12 but not 13-16WP. Once daily Retigabine failed to significantly improved movement distance performance. **B)** Exposure to GW chemicals significantly decreased movement rate scores at both 9-12 and 13-16WP. Retigabine significantly improved rate scores relative to GRP A2 and rescued these scores relative to GRP C2 during treatment (9-12WP}. In the post-treatment phase, movement rates were not improved relative to exposed untreated rats (13-16WP; GRP A2). **C)** Resting duration was unchanged by exposure to GW agents at any observation period. Retigabine significantly increased resting scores at 9-12WP. B: baseline testing; GRP A2: DEET, chlorpyrifos, PB, permethrin; GRP C2 (n=6): (ethanol, corn oil, ethanol, water); GRP R (n=6): DEET, chlorpyrifos, PB, permethrin. Prior to treatment, GRP A2 was composed of 12 rats. During and after treatment GRP A2 was composed of 6 rats. **significantly different by ANOVA.

We had previously shown that $Na_v1.9$ was upregulated 8 weeks following exposure to the 3 agent protocol (Nutter and Cooper, 2014). We have now demonstrated the $Na_v1.9$ exhibited increased activity at 16WP (see below). We used an FDA approved agent Riluzole to examine whether inhibition of this ion channel could improve pain signs in rats exposed to GWI agents. Our laboratory demonstrated that Riluzole can inhibit $Na_v1.9$ current in vascular nociceptors (see Appendix, figure A1. p. 40). In the studies below, we examined whether a 4 week treatment with Riluzole could acutely and/or permanently reverse the pain-like symptoms that developed in our model. As indicated in our SOW, we also examined whether combinations of two treatments (Riluzole and Retigabine) would produce a better outcome.

Riluzole Fails to Ameliorate Pain-Like Signs

Animals were prepared in a similar fashion to examine whether Riluzole (500 mg/kg/day; oral gavage) could also improve pain-like behaviors in our GWI model. Groups were formed and tests were conducted using procedures that were identical with the Retigabine studies presented above. An additional group was treated with combined doses of Riluzole and Retigabine.

Rats treated with Riluzole exhibited only greater reductions of movement distance and increases in resting relative to control rats (figure 2A and 2C). This pattern could have indicated increased pain behavior in these measures or more likely reflected substantial side effects associated with the Riluzole administration. In contrast, rate of movement was not affected by Riluzole (figure 2B). Given that the rate of movement has been our most reliable measure we conclude that there was no beneficial pain outcomes in Riluzole treated rats.

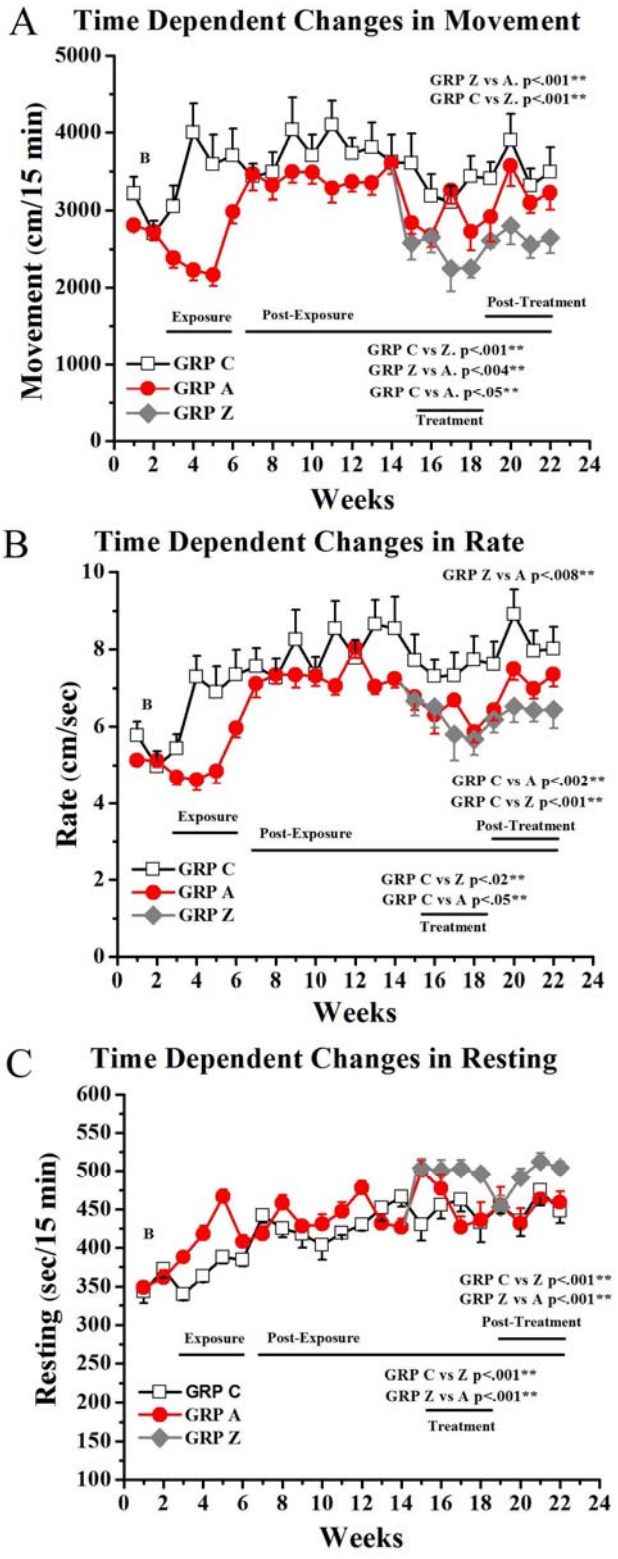

Figure 2. Treatment with Riluzole Depressed All Behavioral Measures. **A)** Exposure to all 4 GW agents (GRP A) significantly decreased movement distance scores at 9-12 but not 13-16WP. Once daily Riluzole (Z) reduced movement distance even further at 9-12WP. These scores remained depressed after Riluzole treatment ceased. **B)** Exposure to GW chemicals also significantly decreased movement rate scores (9-12 and 13-16WP). Riluzole treatment had no influence on rate scores either during treatment (9-12WP) or in the post-treatment phase (13-16WP). **C)** Resting duration was unchanged by exposure to GW agents at any observation period. Regardless, Riluzole significantly increased resting scores at 9-12WP. They remained elevated at 13-16WP. B: baseline testing; GRP A: DEET, chlorpyrifos, PB, permethrin; GRP C (n=6): (ethanol, corn oil, ethanol, water); GRP R (n=6): DEET, chlorpyrifos, PB, permethrin. Prior to treatment, GRP A was composed of 12 rats. During and after treatment GRP A was composed of 6 rats. **significantly different by ANOVA.

In accordance with the SOW, an additional group of rats were treated with a combination of Retigabine and Riluzole (Figures 1 and 2). These studies indicated only increases in pain measures (Figure 3). It was likely that the overall reduction in movement reflected non-specific influences of the combined drug treatment.

Although we originally proposed a treatment test series using a CGRP blocker (BMS-927711) we were unable to execute this portion of the SOW. When the project was submitted in 2014, the CGRP receptor blocker, BMS-927711, was in a stage II clinical trial for use as a migraine treatment. However, it was withdrawn from trials in 2016 due to side effects. We were unable to obtain the compound from Burroughs Welcome or any other vendor despite repeated attempts over a 4 month period. We could not identify another suitable CGRP blocker to use in these studies.

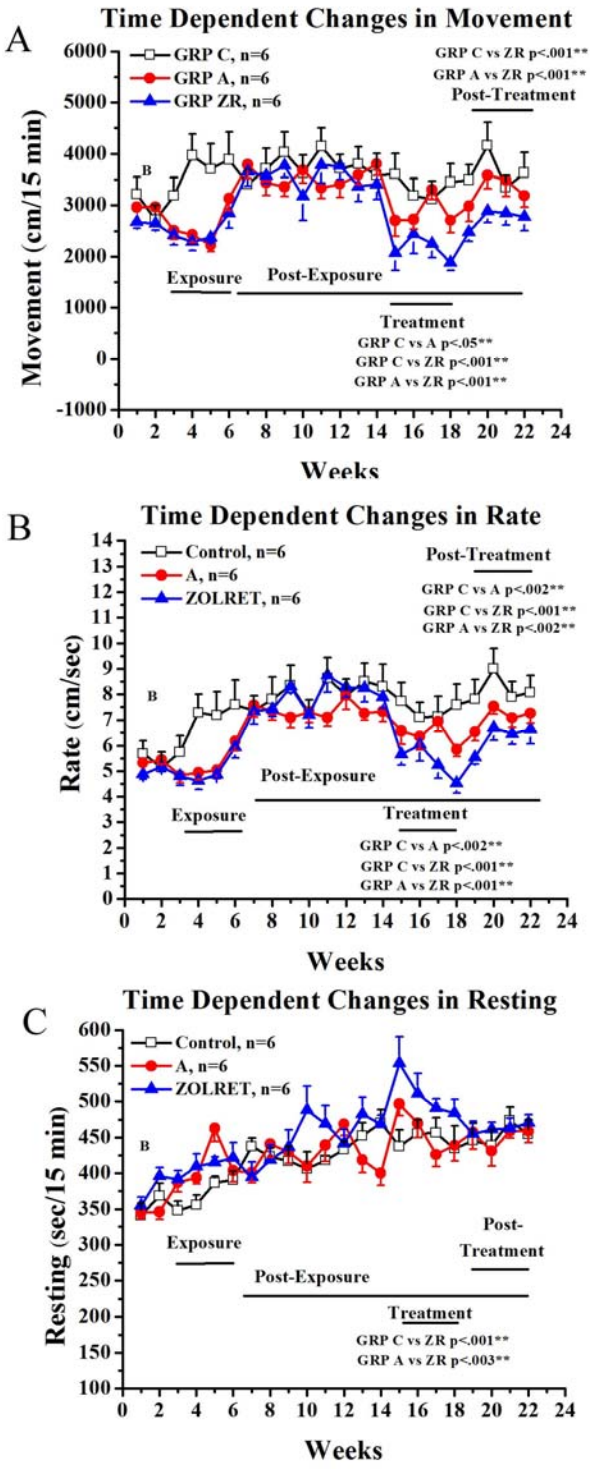

Figure 3. Treatment with Riluzole and Retigabine Depressed All Behavioral Measures. **A)** Exposure to all 4 GW agents (GRP A) significantly decreased movement distance scores at both 9-12WP. Once daily Riluzole and Retigabine (ZR) reduced movement distance even further at 9-12WP. GRP ZR scores remained depressed after ZR treatment ceased. **B)** Exposure to GW chemicals significantly decreased movement rate scores (9-12 and 13-16WP). ZR treatment further decreased rate scores during treatment (9-12WP) and in the post-treatment phase (13-16WP). **C)** Resting duration was unchanged by exposure to GW agents at any observation period. Regardless, ZR significantly increased resting scores at 9-12WP. Rest scored returned to normal levels at the end of the treatment period (13-16WP). B: baseline testing; GRP A: DEET, chlorpyrifos, PB, permethrin; GRP C (n=6): (ethanol, corn oil, ethanol, water); GRP ZR (n=6): DEET, chlorpyrifos, PB, permethrin. **significantly different by ANOVA.

Specific Aim 2. Channel Protein Maladaptations in Myalgic and Arthralgic Rats

TASK 2.1: Assess K_v7 Physiology in Muscle and Vascular Nociceptors 12 and 16 Weeks After Exposure.

Time Line: Months 6-12

We reported outcomes at 12WP (weeks post exposure) in the year 1 annual report. We now report findings at 16 WP. The figures below may also contain 12 WP data that was reported last year.

Rats were prepared and behaviors assessed for these experiments as described above (see also Appendix). As in previous studies, rats were exposed to the 4 GWI chemicals for 4 weeks (optimized protocol). Control rats received only vehicle (corn oil, water, ethanol) exposures. An additional group of rats was prepared that were exposed only to 3 GW chemicals (DEET, permethrin, chlorpyrifos). We had shown in year 1 that rats did not develop pain signs when PB was excluded from the exposure protocol. We use this analytical leverage to determine whether molecular maladaptations were also dependent on PB exposure and therefore clearly related to the presence of pain-signs in rats.

We examined the ambulatory and resting behaviors of rats that were exposed to 3 or 4 GW chemicals (4 weeks; n=38). One group was exposed to DEET, permethrin, chlorpyrifos and PB (Grp A). A second group was exposed to DEET, permethrin, and chlorpyrifos, but PB was excluded (Grp PB). A third group served as a vehicle control (Grp C; water gavage, ethanol topical, corn oil s.c., and ethanol topical).

Behavioral testing was conducted once per week, lasted 15 minutes, and was fully automated by means of an array of infrared beams (AccuScan). AccuScan software converted beam interruptions into measures of ambulation (total distance moved, average rate of movement) and

the total time at rest. Consistent with our previous published work, a repeated measures ANOVA was used to assess ambulation and resting scores captured in 4 week blocks (9-12 weeks and 13-16 weeks post-exposure; 9-12WP and 13-16WP).

Rats exposed to all 4 agents (Grp A) developed enduring pain-like behaviors. Movement distance was significantly reduced at both the 9-12 and 13-16 week post-exposure periods (figure 4A; Grp C vs A). Movement rate was also depressed, but emerged only at the late 13-16WP assessment period (figure 4B; Grp C vs A). Reduced ambulation was accompanied by a significant increase in rest duration at both test periods (figure 4C; Grp C vs A).

Exclusion of PB from the exposure set significantly altered the pattern of behavioral outcomes. In the absence of PB, ambulation deficits were 'rescued' at 9-12WP (movement only) and 13-16WP (figure 4A; Grp C vs Grp PB). This was evidenced as significant increases in movement distance and rate for GRP PB relative to GRP A. It is noteworthy, that, in most comparisons, movement distance and rate scores of Grp PB were not only increased relative to GRP A, but also paradoxically elevated over scores of vehicle-exposed rats (figure 4A and 4B; Grp C vs Grp PB). The absence of PB from the exposure set failed to rescue resting deficits at the 9-12WP assessment period (figure 4C; Grp C vs PB); however, resting deficits were eventually rescued at 13-16WP (figure 4C). At no time were any of the resting scores paradoxically shifted relative to GRP C. This pattern is similar to our published findings of a complex relationship between resting behaviors and GW chemical exposure (Flunker et al., 2017; see also figure 1).

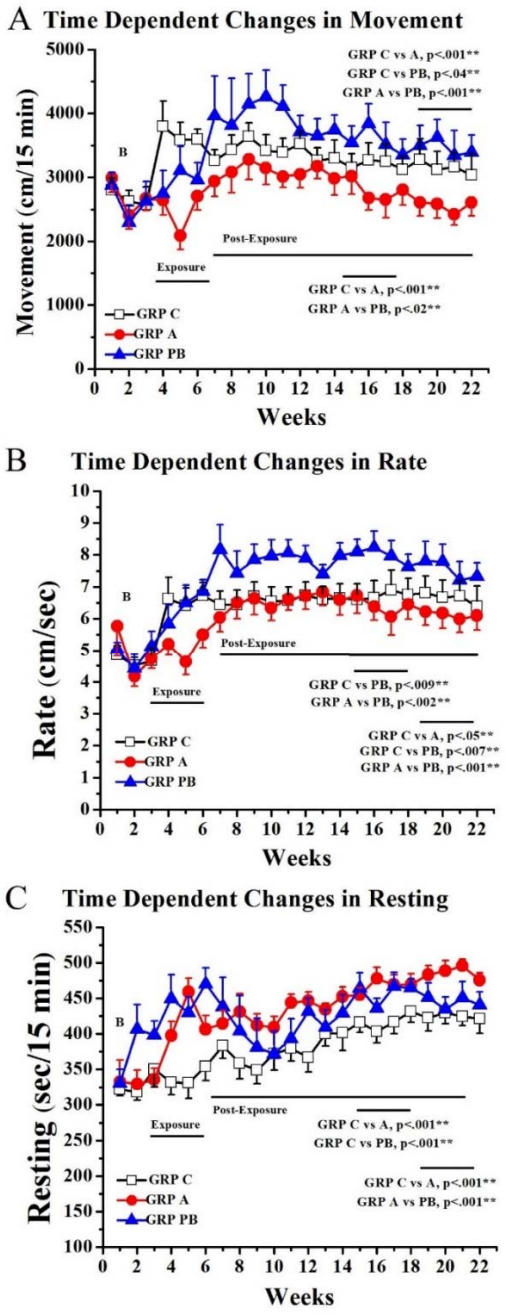

Figure 4. Exposure to 4 GW Chemicals Produces Persistent Pain-Like Behaviors that Required PB. **A)** Exposure to all 4 GW agents (GRP A) significantly decreased movement distance scores at both 9-12 and 13-16WP. When PB was excluded from the exposure set (GRP PB), movement distance was no longer suppressed. **B)** The 4 GW agents also significantly decreased movement rate scores, but declines in rate were delayed until 13-16WP. When PB was excluded from the exposure set, movement rate was significantly increased. **C)** Resting duration was significantly increased at both 9-12 and 13-16WP. In the absence of PB, resting scores were rescued only at 13-16WP. B: baseline testing; GRP A (n=10): DEET, chlorpyrifos, PB, permethrin; GRP C (n=10): (ethanol, corn oil, ethanol, water); GRP PB (n=10): DEET, chlorpyrifos, permethrin. **significantly different by ANOVA.

K_v7 Activity in Muscle and Vascular nociceptors Diverged from Pain Behaviors

We had previously shown that the DEET augmented protocol produced significant shifts in K_v7 conductance that were consistent with increased cellular excitability and reduced ambulatory behavior changes that persisted at 12WP (Flunker et al., 2017). When PB was excluded during the exposure (GRP PB), ambulation was rescued, resting deficits emerged, but K_v7 shifts were retained (12WP; see year 1 report). Therefore, the decline in K_v7 activity diverged from ambulation deficits at 12WP; but might have retained some linkage to resting deficits. We now report that, at 16WP, changes in K_v7 conductance trended in a manner that was not consistent with increased deep nociceptor excitability. While K_v7 activity had returned to normal levels, ambulation and resting deficits were still present at 16WP (figures 4A and B and 5). Accordingly, it did not appear that shifts in K_v7 conductance were critical to either ambulation or resting deficits.

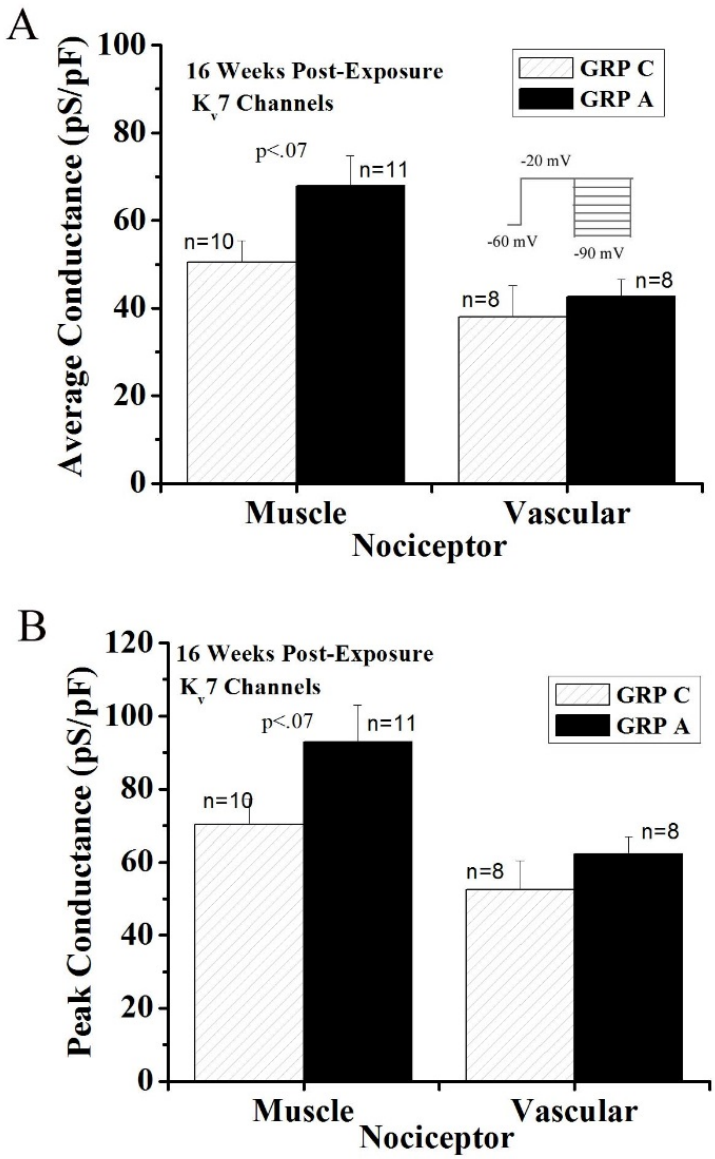

Figure 5. The Average Conductance of K_v7 was Unchanged 16 Weeks Post-Exposure. A) The average conductance was computed from averaged tail current amplitudes that were evoked following a series of stepped repolarizations (-30 to -90 mV, 10 mV steps) from -20 mV. The average conductance was unaffected, but trended strongly upward in muscle nociceptors. An insert, in panel A, presents a diagrammatic representation of the voltage protocol. **B)** The peak conductance was also unchanged, but approached a significant increase in muscle nociceptors at 16WP. Increases in K_v7 activity are not consistent with involvement in GWI pain. GRP A: DEET, permethrin, chlorpyrifos; GRP C: ETOH, ETOH, corn oil, water. Thirteen rats contributed to these experiments (7 GRP C; 6 GRP A).

TASK 2.2 Assess $Na_v1.9$ Physiology in Nociceptors 16 weeks After Exposure

(Figures may also contain 12 WP data that were reported last year)

$Na_v1.9$ Activity in Vascular Nociceptors Parallel Pain Behaviors

Sixteen weeks following exposure to permethrin, chlorpyrifos, PB and DEET, vascular nociceptor physiology was changed in a manner consistent with increased neural excitability and pain (figure 6 and 7). Increased activity of $Na_v1.9$ paralleled behavioral signs of pain during this observation period (figure 4). The hyperexcitable state in the vascular nociceptor pools could have impacts on autonomic function that are consistent with findings of chronic vasodilation in rats exposed to 4 GWI chemicals (see below; figure 8).

Neurons were harvested 16WP exposure to GWI agents. Following cell characterization and series resistance compensation, neurons were exposed to a Na_{iso} solution for 2 minutes (see Appendix). Voltage dependent activation was assessed by application of a series of voltage steps (-80 to -20 mV; 5 mV steps; V_H=-120 mV). Boltzman functions were subsequently fit to the computed values of the peak conductance and plotted against the test voltage series. The voltage of half activation ($V_{.5}$) was determined for each individual cell recording. Cells that failed Boltzman function fits were excluded from the study.

Comparison of computed $V_{.5}$s from control and exposed rats revealed hyperpolarizing shifts in the voltage dependence in vascular nociceptors (figure 6C and D). This outcome indicates that more $Na_v1.9$ current will be evoked as neurons are depolarized and is consistent with increased nociceptor excitability in those rats exhibiting pain-like behaviors. This shift in the $V_{.5}$s could

promote action potential firing during depolarizing events (Copel *et al*., 2009; Herzog *et al*., 2001; Maingret *et al*., 2008; Nutter and Cooper, 2014).

Moreover, when tests were conducted on nociceptors harvested from GRP PB rats (PB excluded from exposure), the $V_{.5}$ values of vascular nociceptors were similar to Grp C, vehicle controls, but significantly *depolarized* relative to GRP A neurons (figure 6E and F). Therefore, the removal of PB from the exposure protocol was associated with rescue of pain-like behaviors, and also rescued the voltage shifts associated with vascular nociceptor excitability (16WP).

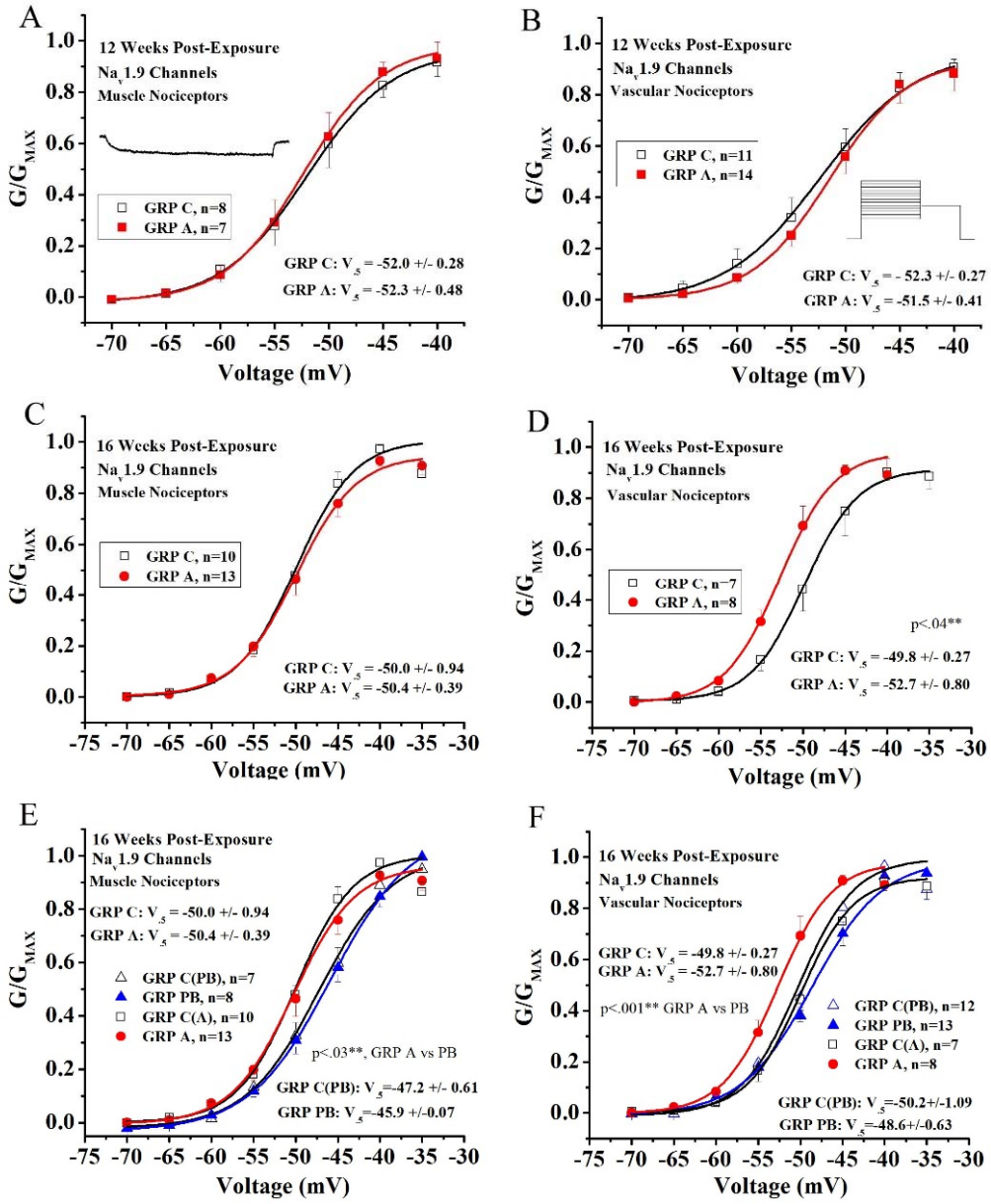

Figure 6. Voltage Dependent Activation of Na$_v$1.9 Shifts with Exposure to 4 GW Chemicals. A and B) Twelve weeks after exposure, the V$_{.5}$ of activation was similar for muscle and vascular nociceptors (GRP C vs GRP A). An insert, in panel A, presents a representative trace that was evoked at -50 mV from a vascular nociceptor (300 msec duration; 1997 pA amplitude). The voltage step protocol is presented as an insert in panel B. **C and D)** Sixteen weeks after exposure, the V$_{.5}$ of activation was hyperpolarized in vascular but not muscle nociceptors (GRP C vs GRP A). **E) and F)** In the absence of PB in the exposure set (GRP PB), the activation V$_{.5}$ was depolarized in both muscle and vascular nociceptors relative to GRP A, but non-different from experiment specific controls (GRP PB vs GRP C(PB)). GRP A: DEET, permethrin, chlorpyrifos, PB; GRP PB: DEET, permethrin, chlorpyrifos; GRP C: ETOH, ETOH, corn oil, water; GRP C(A): control cases for GRP A; GRP C(PB): control cases for GRP PB. Twenty-four rats contributed to these experiments.

We also examined the average evoked $Na_v1.9$ current in muscle and vascular nociceptors. A shift in voltage dependence ($V_{.5}$) predicts that more current will be evoked, but these currents could be independently reduced by other influences on $Na_v1.9$ physiology and result in no net change in excitability. The average evoked current was determined across the active range for each neuron (-65 to -45 mV) and then normalized to the cell dimension parameter capacitance (pF). Statistical comparisons indicated that the average evoked nomalized current amplitudes were significantly increased in vascular nociceptors at 16WP exposure (GRP C vs GRP A, 16 WP; figure 7B). When PB was absent from the exposure protocol, no difference in average currents were detected (GRP C vs GRP PB, 16WP; figure 7C), but the average amplitude of GRP PB currents were significantly reduced relative to GRP A ($p < .001$). These molecular outcomes were consistent with the behavioral outcomes for GRPs A and PB at 16WP and tend to support critical role of vascular nociceptor $Na_v1.9$ in the manifestation of pain-like behaviors that persisted following exposure to GW chemicals.

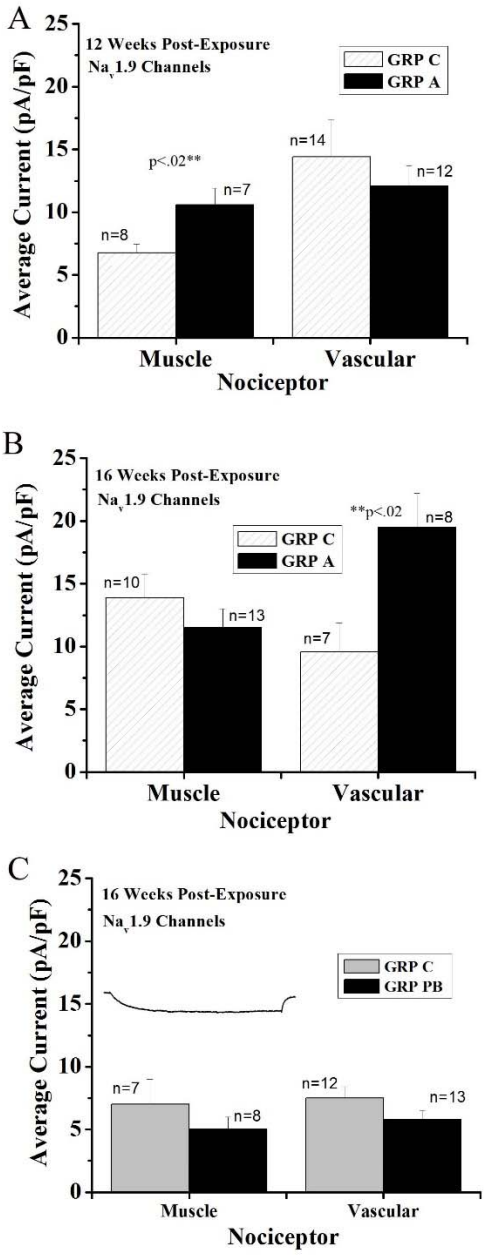

Figure 7. Exposure to GW Chemicals Raises the Average Evoked $Na_v1.9$ Current. A) Twelve weeks after exposure, the average current of muscle nociceptors was significantly higher (GRP C vs GRP A). **B)** Sixteen weeks after exposure, the average current of vascular nociceptors was significantly higher (GRP C vs GRP A), but muscle nociceptors were unaffected. **C)** When PB was excluded from the exposure protocol (GRP PB), the average evoked currents were unchanged in either nociceptor class. An insert, in panel C, presents a representative trace that was evoked at -50 mV from a muscle nociceptor (300 msec duration; 961 pA amplitude). GRP A: DEET, permethrin, chlorpyrifos, PB; GRP PB: DEET, permethrin, chlorpyrifos; GRP C: ETOH, ETOH, corn oil, water. Twenty-four rats contributed to these experiments (see also figure 2; 8 GRP C; 10 GRP A; 6 GRP PB).

TASK 2.4: Assess the Acute Influence of DEET on K_v7 and $Na_v1.9$ Physiology

Timeline: months 1-5

This data was presented in the year 1 report.

TASK 2.3 Assess Excitability and Spontaneous Activity in Nociceptors 12, 16 and 24 weeks After Exposure.

These studies are being conducted as part of the No Cost Extension that is currently in effect.

Specific Aim 3. Autonomic Dysfunction Resulting from GW-Chemical Exposure is Triggered by Hyperactivity in Vascular Nociceptors

TASK 3.1: *In vivo* assessments of changes in hindlimb autonomic vascular reflexes

Timeline: Months 6-15

In these experiments we examined whether vascular reflexes were modified by GW agents. Studies were conducted on rats that received all 4 GW chemicals and on rats that received only DEET, chlorpyrifos and permethrin (PB excluded).

Description: Following exposure to 4 GW-Chemicals, measures of antidromic reflex vasodilation and ipsilateral decentralized nerve stimulation–elicited vasoconstriction were performed in anesthetized, terminal preparations.

Rats in the "Exposed" group were treated with permethrin (2.6 mg/kg; mixture of 26.4% cis and 71.7% trans; Sigma Aldrich), chlorpyrifos (120 mg/kg; Sigma Aldrich), pyridostigmine bromide (PB; 13 mg/kg), and insect repellant DEET (N,N-Diethyl-meta-toluamide, topical; 0-400 mg/kg; daily). A second exposed group of rats were treated in the same manner but with PB excluded from the exposure protocol (GRP PB). Control rats received on vehicle exposures. Application of agents are as described above. All rats underwent blood flow testing prior to and after chemical treatments. Other details of the methods used in these experiments are presented in the Appendix (p. 44).

Consistent with physiology studies that indicated a dysfunction in vascular nociceptors, animals treated with all GWI chemicals (permethrin, chlorpyrifos, PB and DEET, displayed a significantly higher mean blood flow in the hindpaw (figure 8) at up to 12WP. However, despite similar trends, these changes did not persist at 16WP. When PB was excluded during the exposure, no changes in blood flow were observed in any test period (figure 8). Interestingly, the increased vasodilatation in chemically exposed animals was not due to an increase in autonomic cardiovascular measures during that period. Accordingly, no significant differences in exposed versus control measurements of systolic blood pressure, pulse rate, core body temperature, and paw skin temperature were observed during the post-exposure period (see year 1 report). This suggests the increased vasodilation may have been due to overactive vascular afferents releasing vasodilator transmitter, like CGRP. In a separate group of animals exposed to only 3 GWI chemicals (minus PB), the increased vasodilatation in the hindpaws did not occur (figure 8). The absence of any vascular effects in the group of rats that were not exposed to PB links these autonomic changes to those behavioral pain-like signs that showed a similar PB dependence (figure 4).

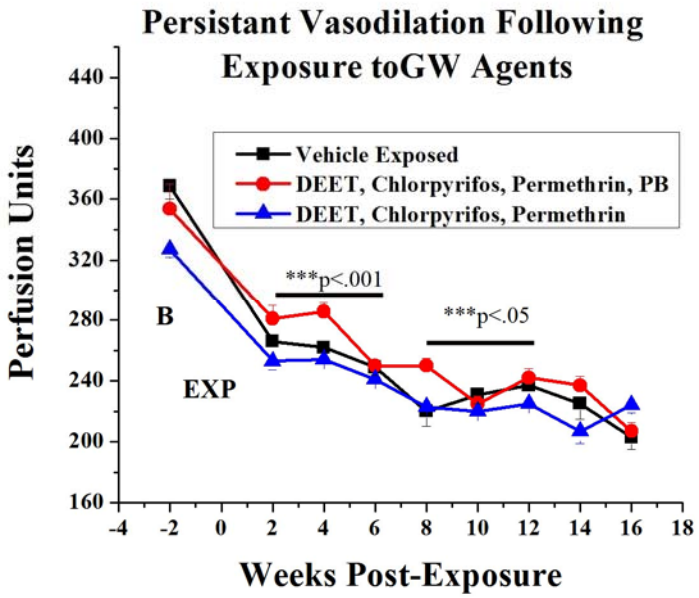

Figure 8. Exposure to GWI chemicals produces increased vasodilatation. A) Rats exposed to GW agents for 4 weeks develop persistent vasodilation ($p<.001$, weeks 2-6; $p<.05$ weeks 8-12). When PB is absent from the exposure, vasodilation is significantly reduced relative to 4 GW agent exposed rats ($p<.001$ at weeks 2-6 and 8-12). Laser speckle contrast imaging of hindpaw (n=6). B: baseline; EXP: 4 week exposure to PB and/or permethrin, chlorpyrifos, and DEET. No measures were taken during the 4 week exposure.

TASK 3.2: *In vivo* assessments of spontaneous activity in vascular nociceptors

TASK 3.3: *In vivo* assessments of the efficacy of treatment with CGRP blocker on autonomic measures

These studies were scheduled for year 2 (month 18) and are now being completed as part of the no cost extension.

4. Impact

Impact on Principal Disciplines

Our rat model of GWI pain produces robust and highly reproducible changes in rat ambulation that are consistent with the presence of a chronic pain condition. It also produces autonomic signs that are similar to those reported in GW veterans (Haley et al., 2009; Liu et al., 2011; Li et al., 2011). Both behavioral and autonomic signs were critically dependent on the inclusion of PB (and chlorpyrifos) in the exposure protocol.

These behavioral outcomes have been replicated 7 times (with and without DEET augmentation) over the course of our CDMRP funding. The pain behaviors are sometimes delayed in their expression (figure 1) and sometimes develop rapidly (figure 2). Both patterns were reported in GW veterans (Kroenke et al., 1998). We have shown that these signs can persist up to 24 weeks after exposure to GW chemicals ended (Flunker et al., 2017). We have also shown that DEET was not a necessary component but greatly accelerated the development of pain signs and increased

their magnitude. Much of this data was first reported in year 1 and has been replicated in year 2 (see also Flunker et al., 2017).

We have now shown that certain molecular correlates of exposure to GW chemicals do not directly relate to the appearance and persistence of pain behaviors (K_v7), but in fact diverge from these behaviors, both temporally (at 16WP) and with the exclusion of PB from the exposure set. It is therefore unlikely that K_v7 is fundamental to the pathophysiology of the syndrome. Nevertheless, utilization of a K_v7 active agent produced reversible reductions in pain behaviors during their administration (figure 1). This indicates that manipulation of this ion channel could be an effective palliative for certain aspects of the condition (pain), but is unlikely to provide relief from other symptoms (cognitive, motor, autonomic). FDA approved K_v7 openers are available and have utility in other chronic pain conditions (Devulder, 2010). Further development of this approach is indicated, including the use of other compounds (e.g. flupirtine), higher dosages, multiple doses per day, and longer treatment periods.

We further demonstrated that $Na_v1.9$ activity did co-vary with the presence of pain signs at the 16WP interval. Removal of PB from the exposure set eliminated changes in $Na_v1.9$ activity and behavioral pain signs. Our studies using, Riluzole, an FDA approved agent that inhibits $Na_v1.9$ and other persistent Na^+ channel currents (Desaphy et al., 2013; Fehlings et al., 2012) produced only substantial side effects in our studies. Possibly the doses were too high.

The changes in $Na_v1.9$ activity were limited to vascular nociceptors (Type 8; Petruska et al., 2000; 2002; Rau et al., 2014). Alterations in vascular nociceptor functions are consistent with persistent

vasodilation we have observed in GW chemical exposed rats and with the presence and absence of PB in the exposure set (figure 8). These outcomes suggest hyperactivity in vascular nociceptors consequent to the release of vasodilator CGRP and possibly NO (nitric oxide), as well. Nitric oxide can impact nociceptor function via interaction with TRPA1 (Aubdool et al., 2014; Eberhardt et al., 2014; Nassini et al., 2014). Some type 8 nociceptors express TRPA1, but many do not (unpublished observations). Perhaps this subgroup plays an important role in GWI pathophysiology.

We have shown that these type 8 vascular neurons express both CGRP and SP (Substance P; Rau et al., 2014). Release of CGRP from vascular nociceptors has both central and peripheral nervous system impacts on chronic pain. Migraine headache has been linked to vasodilation and CGRP release (Durham, 2010) and headache is one of a constellation of symptoms associated with GWI. Our efforts to use BMS927711, an orally effective CGRP blocker that was in clinical trials at the time this application was made, were frustrated when this drug was removed from development due to the development of significant side effects (Marcus et al., 2014). No substitute was available for testing. Perhaps new drugs that block CGRP function will become available in the coming years.

<u>There was no impact on technology transfer-nothing to report</u>

<u>There was no impact on society-nothing to report</u>

5. Changes/Problems

Due to its removal from the market, we were unable to obtain BMS-927711 for testing purposes. Therefore, portions of TASK 1 could not be executed.

6. Products

Journal Publications

The following manuscript was published:

Flunker, LK, Nutter TJ, Johnson, RD and Cooper, BY. DEET Potentiates the Development and Persistence of Anticholinesterase Dependent Chronic Pain Signs in a Rat Model of Gulf War Illness Pain. Toxicology and Applied Pharmacology, 2017, 316: 48-62

Abstracts and Presentations

The following abstract was presented at the Society for Neuroscience 2016.

Cooper, B.Y., Nutter, T.J., Johnson, R.D and Flunker, L. Contributions of DEET to a Rat Model of Gulf War Illness Pain

Introduction. Veterans of the 1991 Gulf War commonly reported a delayed onset joint, muscle and other deep tissue pain. The Research Advisory Committee on Gulf War Illness (GWI) has determined that pesticides may have contributed to the development of the symptoms of GWI (Binns et al., 2008). We developed a rat model of GWI pain based upon a 60 day exposure to permethrin (P), chlorpyrifos (CP) and pyridostigmine bromide (PB; Nutter et al., 2015). In the present report, we combined behavioral and molecular approaches to examine the contribution of DEET to the development of the joint and muscle pain of GWI.

Methods. Juvenile male rats, weighing between 90 and 110 g, were exposed to various combinations of P (2.6 mg/kg; topical), CP (120 mg/kg; subcutaneous (SC)), PB (13 mg/kg; oral gavage), and DEET (400 mg/kg; topical) for 30 days. Using an identical administration schedule, control group rats received only vehicle exposures (topical ethanol, SC corn oil, water by gavage). All rats underwent behavioral testing before, during and after chemical exposures (hindlimb pressure withdrawal; open field activity (movement distance, movement rate and resting duration). Molecular studies were conducted to assess the influence of acute DEET on nociceptors. In

molecular studies, young adult rats weighing 90-150 grams were anesthetized and decapitated. Whole cell clamp experiments were conducted on excised dorsal root ganglion neurons that were identified as muscle or vascular nociceptors using the method of Scroggs and Cooper (Cardenas et al., 1995; Petruska et al., 2002).

Results. When exposed to all 4 compounds, rats exhibited reduced open field activity (movement distance and rate) that resembled a myalgia or arthralgia 9-12 weeks after dosing had ceased ($p<.02$ and $p<.004$). When exposed to only 3 compounds, activity changes failed to materialize in the absence of PB or CP but persisted in the absence of permethrin (movement; $p<.05$); moreover, when PB was removed, rate decreases were significantly lessened relative to exposure to all 4 chemicals ($p<.05$). Molecular studies indicated that DEET significantly inhibited $Na_v1.9$ amplitude ($p<.04$; vascular nociceptors), but had no effect on K_v7 or $Na_v1.8$. The influence DEET on $Na_v1.9$ only occurred at relatively high doses that are not likely *in vivo* (100 μM).

Conclusions. DEET makes a significant contribution to a robust deep tissue pain syndrome in a rat model of GWI pain. PB was required for, and CP contributed to, motor activity changes while permethrin did not play a role at 12 weeks-post exposure. DEET might exert its influence through inhibition of $Na_v1.9$.

Accepted for Presentation at Society for Neuroscience 2017:

Molecular maladaptations to vascular nociceptor $Na_v1.9$ covaries with exposure to pyridostigmine bromide in a rat model of Gulf War Illness pain

Cooper, B.Y., Nutter, T.J.., Flunker, L.K. and Johnson, R.D

Introduction. Many veterans of Operation Desert Storm (ODS) still struggle with symptoms of GWI. Symptoms are manifested as diverse cognitive, motoric and sensory abnormalities that include chronic pain. In order to understand the pathophysiology of GWI pain, our laboratory has developed rat models of this multisymptom disorder.

Method. We examined the influence of 4 GW agents on the ambulatory and resting behaviors of rats. Young adult male rats were exposed to either 3 or 4 GW chemicals for a period of 4 weeks. One group was exposed to DEET (400 mg/kg; topical, 50%), permethrin (2.6 mg/kg; topical), chlorpyrifos (120 mg/kg; s.c.) and pyridostigmine bromide (PB, 13 mg/kg; oral; GRP A, n=56). A second group received the same exposure but PB was excluded from the protocol (GRP PB, n=10). A third group served as a vehicle control (Grp C, n=28; ethanol topical, corn oil s.c., and water gavage). Ambulation and resting scores were measured weekly by an automated infrared detection system. Nine weeks after exposure, thirty rats received treatments intended to ameliorate deep tissue pain (Riluzole, 3 mg/kg; Retigabine, 7 mg/kg; 14 days). Sixteen weeks after chemical exposures ended, an additional 34 rats were euthanized, and their dorsal root ganglia prepared for whole cell patch studies. ANOVA was used to assess changes in rat behaviors due to exposures and treatments. Student t tests were used to assess molecular data.

Results. Rats exposed to 4 GW chemicals (GRP A) developed pain-like deficits in ambulation and resting that persisted 13-16 weeks post-exposure (16 WP; $p<.001$ and $p<.001$, respectively). Rats exposed to only 3 GW agents (PB excluded) did not exhibit pain-like signs in weeks 13-16. Compared to vehicle exposed rats (GRP C), the amplitude of vascular, but not muscle nociceptor $Na_v1.9$ was elevated in GRP A (16WP; $p<.02$); but K_v7 activity was unchanged. When PB was excluded from the exposure (GRP PB) vascular nociceptor $Na_v1.9$ amplitude was similar to controls and significantly reduced relative to neurons harvested from GRP A rats (16 WP; $p<.005$); Treatment with $Na_v1.9$ inhibitor, Riluzole, did not improve behavior scores at 9-10WP; but K_v7 opener Retigabine did produce positive trends ($p<.07$).

Conclusion. Exposure to PB was critical for the emergence of persistent pain signs in a rat model of GWI. Maladaptations of vascular, but not muscle nociceptor, $Na_v1.9$, covaried with the manifestation of pain-like behaviors. Treatments targeting $Na_v1.9$ were not effective at 10WP.

Long-term increases in hindlimb vasodilatation following exposure to Gulf War Illness (GWI) chemical prophylactic agents is independent of cardiovascular parameters and suggests involvement of CGRP release from vascular nociceptor endings in a rat model of GWI pain

Tournade, CM, Nguyen, HD, Cooper, BY, Johnson, RD

Gulf War veterans experienced high levels of exposure to insecticides/repellants and nerve gas chemoprophylactic agents, which resulted in a series of chronic clinical symptoms (i.e. Gulf War Illness). These symptoms include unusual complexes of headache, joint, muscle and abdominal pain. We previously developed a rat model of GWI pain produced by a 4-week exposure to 3 or 4 GWI chemical agents, measurements of ambulatory/resting behaviors during the exposure and the 16W post exposure period, and determination of electrophysiological profiles of single vascular nociceptors. In the present study, we hypothesized that GWI chemical-induced maladaptations in vascular afferents (shown by our previous data) would produce increased peripheral release of their predominant constitutive vasodilatory neuropeptide, CGRP, resulting in subsequent vasodilatation and increased detectable blood flow. This study used a noninvasive laser scanning contrast imaging (LSCI) to measure hindpaw blood flow. Young adult male rats were exposed to either (i) DEET (400 mg/kg; topical, 50%), permethrin (2.6 mg/kg; topical), chlorpyrifos (s.c.; 120 mg/kg) and pyridostigmine bromide (PB,13 mg/kg; oral; GRP A, n=16), (ii) all chemicals except for PB (GRP PB, n=14), or (iii) vehicle control (Grp C, n=16; ethanol topical, corn oil s.c., and water gavage). Before and every two weeks after chemical exposure, rats were briefly anesthetized with isoflurane (10-15min) and blood flow in the plantar hindpaws recorded for five minutes with a PeriCam LSCI laser probe. The analysis area was set by programmable software based on spatial landmarks and was used for all animals to validate inter-animal and within-animal comparisons. Cardiovascular parameters of tail cuff blood pressure (systolic and mean arterial pressures), heart rate, and temperature (body core and hindpaw) were also measured. There were highly significant increases in hindpaw blood flow (vasodilatation) at post exposure weeks 4-10 in GRP A rats compared to GRP C controls, corresponding to behavioral measures of pain, despite the lack of differences in cardiovascular measures of blood pressure, heart rate, and temperature. Interestingly, the GRP PB rats that lacked exposure to PB, did not show increased blood flow during the post exposure period. We conclude that in animals exposed to the four GW chemicals, long-term increases in blood flow (vasodilatation) were produced for at least 8 weeks after the exposure period, and in the absence of changes in cardiovascular parameters, suggests that hyperactivity in vascular afferents leads to increased release of vasodilator neuropeptides (e.g. CGRP) from the terminal endings.

Inventions, patent applications, and/or licenses

None

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

Personnel:

Name: Brian Y. Cooper, Ph.D., College of Dentistry

Project role: Principal Investigator,

Researcher Identifier (ORCID ID): 0000-0002-7592-588X

Nearest person month worked: 6

Contribution to Project:

Design, execution and analysis of physiology and behavior experiments (Tasks 1.2, 1.3, 2.1, 2.4)

Preparation of manuscripts and abstracts

Preparation of IACUC protocols

Name: Richard D. Johnson, Ph.D., College of Veterinary Medicine

Project role: Co-Principal Investigator

Researcher Identifier: none

Person Months: 3

Contribution to the Project:

Design, execution and analysis of physiology experiments (Tasks 3.1, 3.2, 3.3)

Preparation of manuscripts and abstracts

Preparation of IACUC protocols

Thomas J. Nutter, Ph.D., College of Dentistry

Project Role: Biological Scientist

Researcher Identifier: none

Person Months: 12

Contribution to Project:

Execution of physiology experiments (Tasks 1.2, 1.3, 2.2)

Linda Flunker, MS, College of Dentistry

Role on Project: Biological Scientist

Research Identifier: none

Person Months: 11

Contribution to Project:

Execution of Behavioral Studies (Tasks 1.2, 1.3, 2.1, 2.2, 2.3, 3.1, 3.2, 3.3; dosing; activity measures; data collection and storage)

Funding Support: Ms Flunker is assigned 90% to the project and 10% to departmental projects

Name: Victoria Dugan, College of Veterinary Medicine

Role on Project: Biological Scientist

Researcher Identifier: none

Person Months: 6

Contribution to Project:

Execution of physiology experiments (Tasks 3.1, 3.2, 3.3)

Funding Support: Ms Dugan is assigned 50% to the project and 50% to NIH projects

Change in the Support for the PI:

Nothing to report

Partner Organizations:

Nothing to report

Changes/Problems**:**

Nothing to report

8. Special Reporting Requirements

none

9. Appendices

Supplementary Figures

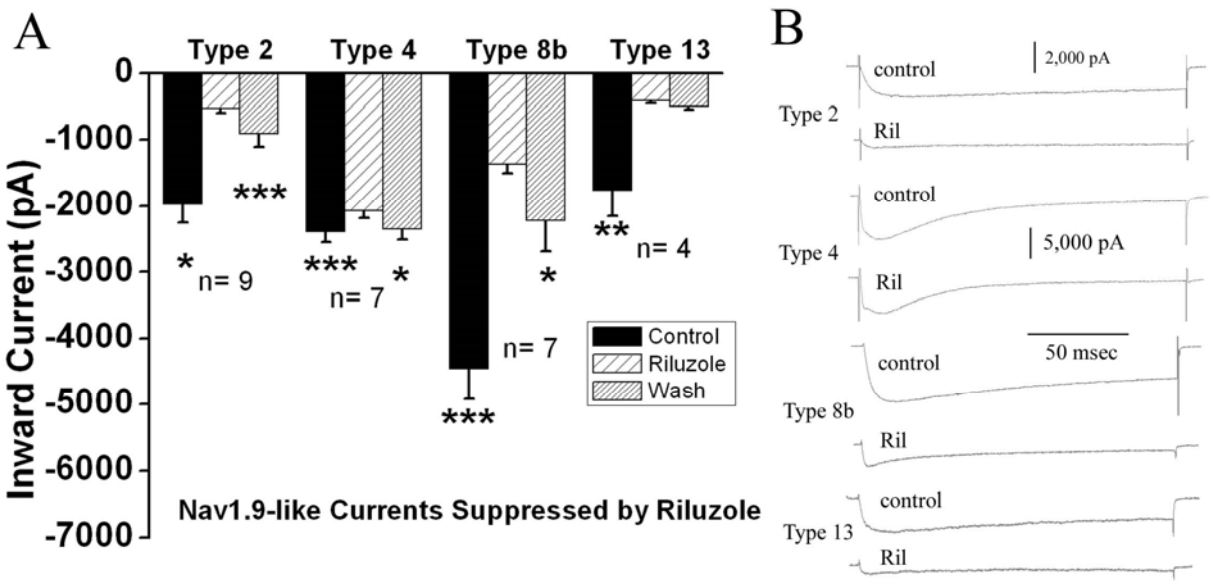

Figure A1. Vascular Nociceptor Nav1.9 Currents were Inhibited by Riluzole. A) Type 8 vascular nociceptors $Na_v1.9$ is significantly inhibited by Riluzole (10 uM; $p<.01$). The current recovers after a 2 minute wash out of Riluzole ($p<.05$). **B)** Representative $Na_v1.9$ current traces in superficial and deep tissue nociceptors (skin: types 2, 4 and 13; vascular nociceptor: type 8).

Statement of Work

Research TASKS	Rats	Months	University of Florida
Specific Aim 1. Reversing Signs of GWI Pain Behaviors Maintained by Vascular Nociceptors			
TASK 1.1: Optimize the Chemical Exposure Protocol	50	1 to 5	Beh: Dr. Cooper
Description: We will determine whether all 4 GWI chemicals (Permethrin, Chlorpyrifos, PB and DEET) are required			Autonomics: Dr. Johnson
to produce the persistent pain behavior and autonomic dysfunction complex			
Methods: Rats are exposed to 3 or 4 GWI chemicals for 4 weeks (see Table 1, project narrative).			
Behavioral measures of pain and autonomic functions are performed (pain measures weekly;			
autonomic measures bi-monthly).			
Milestone: Necessary conditions are established for a pain and autonomic disorder (reduced muscle pain threshold,			
decreased motor activity, increased resting, LSCI score, see TASK 3.1).			
TASK 1.2: Targeting Maladapted Ion Channel Proteins with Systemic Treatments			
Description: Once ion channel protein treatment targets are identified (Specific Aim 2), we will use agents	72	14 to 19	Beh: Dr. Cooper
that modulate these proteins (e.g., retigabine, riluzole, BMS-927711) to reverse signs of an			Autonomics: Dr. Johnson
established pain and autonomic disorder that are present 4-8 weeks after the chemical exposure			
has ended. Dose effects are examined within the 4-8 week window. The time course of			
successful treatments will be characterized. The side effects of successful treatments are			
evaluated. Male and female rats are used during tests			
Milestone A. Measures of pain and autonomic disorders are reduced significantly 2 hours following			
treatment (normalized muscle pain threshold, motor activity, resting, LSCI score, see TASK 3.1).			
Milestone B. Reduced pain and autonomic measures are maintained for 4 weeks following treatment			
TASK 1.3: Targeting Maladapted Ion Channel Proteins with Multiple Systemic Treatments	48	20 to 24	Beh: Dr. Cooper
Description: If single agent treatments fail to resolve the pain and autonomic behavior complex,			Autonomics: Dr. Johnson
we will examine the efficacy of multiple agents that target different proteins and may differentially influence			
pain versus autonomic signs.			
Milestone A. Measures of pain and autonomic disorders are reduced significantly 2 hours following			
treatment (normalized muscle pain threshold, motor activity, resting, LSCI score, see TASK 3.1).			
Milestone B. Reduced pain and autonomic measures are maintained for 4 weeks following treatment.			

Research TASKS	Rats	Months	University of Florida
Specific Aim 2. Channel Protein Maladaptations in Myalgic and Arthralgic Rats	**Rats**	**Months**	**University of Florida**
TASK 2.1: Assess K_v7 Physiology in Muscle and Vascular Nociceptors 12 and 16 Weeks After Exposure.	29 + 15	6 to 12	Phys: Dr. Cooper
Description: Rats are exposed to the optimized GW chemical protocol for 4 weeks. Behavioral measures of			Beh: Dr. Cooper
pain and autonomic functions are performed (pain measures weekly; autonomic measures bi-			Autonomics: Dr. Johnson
monthly). Cells are harvested from exposed rats 12 and 16 weeks after exposure (24 rats).			Phys: Dr. Johnson
Studies are conducted on vascular and muscle nociceptors. Whole cell voltage clamp			
electrophysiology is performed. Measures of voltage dependence, current amplitude and			
kinetics are assessed. Behavioral testing continues out to 24 weeks (20 rats).			
Milestone A: K_v7 amplitude is significantly decreased relative to vehicle treated controls			
12 and 16 weeks after exposure			
Milestone B: Pain behavior and autonomic signs are maintained for 24 weeks			
TASK 2.2 Assess $Na_v1.9$ Physiology in Nociceptors 12 and 16 weeks After Exposure	29 + 15	6 to 12	Phys: Dr. Cooper
Description: Rats are exposed to the optimized GW chemical protocol for 4 weeks. Behavioral measures of			Beh: Dr. Cooper
pain and autonomic function are performed (pain measures weekly; autonomic measures bi-monthly).			Autonomics: Dr. Johnson
Cells are harvested from exposed and vehicle treated rats 12 and 16 weeks (24 rats)			Phys: Dr. Johnson
following exposure. Whole cell voltage clamp electrophysiology is performed. Studies are			
conducted on vascular and muscle nociceptors. Measures of voltage dependence, current			
amplitude and kinetics are assessed. Behavioral testing continues out to 24 weeks (20 rats).			
Milestone A: $Na_v1.9$ amplitude is significantly increased relative to vehicle treated controls 12 and 16			
weeks after exposure.			
Milestone B: Pain behavior and autonomic signs are maintained for 24 weeks.			
TASK 2.3 Assess Excitability and Spontaneous Activity in Nociceptors 12, 16 and 24 weeks After Exposure.	36	14 to 20	Phys: Dr. Cooper
Description: Rats are exposed to the optimized GW chemical protocol for 4 weeks. Behavioral measures of			Beh: Dr. Cooper
pain and autonomic function are performed (pain measures weekly; autonomic measures			Autonomics: Dr. Johnson
biweekly). Cells are harvested from exposed and vehicle treated rats 12, 16 and 24 weeks			
following exposure. Whole cell current clamp electrophysiological studies are performed			
Studies are conducted on vascular nociceptors. Measures of spontaneous activity			
and excitability are assessed at room temperature and 35° C.			
Milestone A: Action potential excitability is significantly increased relative to vehicle treated controls			
Milestone B: Action potential spontaneous activity is significantly increased relative to vehicle treated controls			
TASK 2.4: Assess the Acute Influence of DEET on K_v7 and $Na_v1.9$ Physiology	48	1 to 5	Phys: Dr. Cooper
Description: The addition of DEET to the exposure protocol is essential to establishing a persistent pain			
behavior complex. The pathway to this outcome is unknown. We will assess the acute			
influence of DEET (100-600 μM) on the physiology of $Na_v1.9$ and K_v7 channel proteins. Whole			
cell voltage clamp electrophysiology is performed on young adult rats. Studies are conducted on			
vascular and muscle nociceptors. Measures of voltage dependence, current amplitude and			
kinetics are assessed			
Milestone A: K_v7 current amplitude is significantly decreased by DEET.			
Milestone B: Nav1.9 current amplitude is significantly increased by DEET			

Research TASKS	Rats	Months	University of Florida
Specific Aim 3. Autonomic Dysfunction resulting from GW-Chemical Exposure is Triggered by Hyperactivity in Vascular Nociceptors	**Rats**	**Months**	**University of Florida**
TASK 3.1: *In vivo* assessments of changes in hindlimb autonomic vascular reflexes	30	6 to 15	Phys: Dr. Johnson
Description: Following optimized GW-Chemical exposure (Task 2.1) and behavioral evidence of myalgia,	same as 2.1		
LCSI measures of antidromic reflex vasodilation and ipsilateral decentralized sympathetic trunk			
stimulation–elicited vasoconstriction will be taken in anesthetized,			
terminal preparations. Post-exposure periods will be 12W (n=12) and			
16W (n=12) along with 6 saline-controls at each time point			
Milestone A: GWI-Chemical exposure increases antidromic reflex vasodilatation in gastrocnemius muscle.			
Milestone B: GWI-Chemical exposure decreases sympathetic stimulation-induced vasoconstriction			
TASK 3.2: *In vivo* assessments of spontaneous activity in vascular nociceptors	30	6 to 15	Phys: Dr. Johnson
Description: Following optimized GW-Chemical exposure (Task 2.2) and behavioral evidence of myalgia,	same as 2.2		
levels of spontaneous activity in single vascular nociceptive			
afferent fibers will be measured in anesthetized, terminal preparations.			
Post-exposure periods will be 12W (n=12) and 16W (n=12) along with 6 saline-controls at each time point			
Milestone A: GWI-Chemical exposure increases spontaneous activity in vascular nociceptors			
TASK 3.3: *In vivo* assessments of the efficacy of treatment with CGRP blocker on autonomic measures	12	14 to 19	Phys: Dr. Johnson
Description: Following optimized GW-Chemical exposure (Task 1.2) and 4 weeks after treatment with CGRP blockers,	same as 1.2		
assessments of spontaneous activity in single vascular nociceptive afferent fibers and hindlimb autonomic			
vascular reflexes will be measured in anesthetized, terminal preparations, to determine if autonomic vascular reflexes			
and spontaneous activity measures are normalized. Post-exposure periods will be 4W (n=6) along with 6 saline-controls			
Milestone A: GWI-Chemical exposure induced changes in autonomic and vascular afferent-mediated reflexes are			
reduced or normalized after CGRP blocker treatment.			

Research TASKS	Preparation Grant Month	Submission Grant Month	University of Florid
TASK 4: Data Reduction and Dissemination			
TASK 4.1: Optimized Protocol	6	Combined with Physiology	Cooper Laboratory
TASK 4.2: Kv7 Measures (TASK 2.1)	12	15	Cooper Laboratory
TASK 4.3: Nav1.9 Measures (TASK 2.2)	12	15	Cooper Laboratory
TASK 4.4: Acute DEET on Kv7 and Nav1.9 (TASK 2.4)	6	9	Cooper Laboratory
TASK 4.5: Spontaneous Activity and Excitability (TASK 2.3)	18	24	Cooper Laboratory
TASK 4.6: Targeted Treatments (TASKs 1.2 and 1.3)	18	24	Cooper Laboratory
TASK 4.7: In vivo autonomic vascular reflexes (TASK 3.1)	18	24	Johnson Laboratory
TASK 4.8: In vivo spontaneous activity in vascular nociceptors (TASK 3.2)	18	24	Johnson Laboratory
TASK 4.9: In vivo efficacy of CGRP blockers on autonomic measures (TASK 3.3)	18	24	Johnson Laboratory

Methods

Subjects

Fifty (80) young adult male rats were used in the pesticide exposure studies (Sprague-Dawley; Envigo/Harlan). Rats entering the study weighed 90-110 grams. All animals were housed in American Association for Accreditation of Laboratory Animal Care approved quarters, and all procedures were reviewed and approved by the local Institutional Animal Care and Use Committee and ACURO (Animal Care and Use Review Office of the Army Medical Research and Materiel Command). Two rats developed health issues and were euthanized. After chemical exposures had ended, one rat manifested a rigidity of one hindlimb and the second rat developed a ventral midline tumor. There were no signs of acute pesticide toxicity typically associated with permethrin or chlorpyrifos during the execution of these studies.

Chronic Exposure Protocol

Over a period of 4 weeks, 50 rats were exposed to permethrin (2.6 mg/kg; mixture of 26.4% cis and 71.7% trans; Sigma Aldrich), chlorpyrifos (120 mg/kg; Sigma Aldrich), DEET (200 or 400 mg/kg; Sigma Aldrich) and pyridostigmine bromide (PB; 13 mg/kg; Sigma Aldrich). Permethrin, in ETOH, was applied every day to a shaved area of the back (~1 square inch) between the forelimbs. Chlorpyifos was administered by a subcutaneous injection (corn oil) once every 7 days. The dose of chlorpyrifos was intended to represent a net exposure to the potentially large and varied anticholinesterases that soldiers were exposed to in the Gulf theater (Binns et al., 2008). Chlorpyrifos was administered in a corn oil formulation that released the agent over a couple of days (Smith et al., 2009). DEET was administered topically in ethanol. PB was administered daily

by oral gavage (tap water) based upon a standard military dose that was adjusted to account for faster pharmacokinetics in rodents (Birtley et al 1966; Husain et al., 1968; Aquilonius et al., 1980; Breyer-Pfaff et al., 1985). An additional 10 rats (GRP PB) received only 3 GW agents (permethrin, chlorpyrifos, DEET). Times and methods of dosing were identical to other GW exposed rats. Twenty rats served as controls. They received only vehicle applications (ethanol, water, corn oil). Rats were weighed once per week throughout the studies and doses were adjusted accordingly. Control rats received only vehicle exposures over the identical time course.

Retigabine (1200 mg/kg/day) and Riluzole (500 mg/kg/day) were administered by oral gavage in DMSO. Control groups in these studies received only DMSO. Treatments were administered in the morning and behavioral testing occurred within 2 hours. Treatments continued daily for 4 weeks. Behavioral tested occurred once per week and continued 4 weeks after treatments ceased.

Assessment of Pain Behaviors

Prior to entering the study, rats were acclimated to the behavioral procedures for 2 weeks. Pain assessments were conducted weekly throughout the entire dosing and post-dosing periods. Activity levels (movement distance, average movement rate, and rest time duration) were recorded automatically by infrared sensors in a modified activity box (15 min test period; Fusion Systems, AccuScan Instruments Inc.). The 35 by 40 cm test chamber was modified to prevent rearing behaviors. The chamber was cleaned after each 15 minute test period. Behavioral tests were conducted on both chemically exposed (permethrin, chlorpyrifos, DEET, PB) and vehicle treated (ETOH, corn oil, water) animals over an identical time course. Rats were tested once per week on the behavioral tasks.

Whole Cell Patch Clamp Electrophysiological Studies

Preparation of Cells

Dorsal root ganglion neurons (DRG) were harvested from young adult male rats (90-150 grams). Rats were anesthetized (Isoflurane) and rapidly euthanized by decapitation (Harvard Instruments). The spinal column was removed, bisected and the DRG were dissected free from T11 to S1. Ganglia were trimmed, cut into strips and digested in Tyrode's solution containing collagenase A (2 mg/ml; Roche Chemical) and Dispase II (5 mg/ml; Roche Chemical). A 15 ml centrifuge tube containing the dissected ganglia was placed in a heated, shaking water bath for 90 minutes at 35° C (EDVOTEK Digital Shaking Water Bath). Gentle trituration was then used to break up visible strips of ganglia. The dispersed neurons were then digested for an additional 45 minutes, and then spun at 500 RPM (30 sec). The supernatant was discarded. The remaining pellet was dispersed into 2 ml of Tyrode's, triturated and plated on 9, 35 mm, polylysine coated Petri dishes (Fluorodish). Plated neurons were bathed continuously in a Tyrode's solution, containing (in mM) 140 NaCl, 4 KCl, 2 $MgCl_2$, 2 $CaCl_2$, 10 glucose, and 10 HEPES, adjusted to pH 7.4 with NaOH. All electrophysiological studies were conducted at room temperature (20 °C) within 10 hours of plating. Only one cell was used per Petri dish. Electrodes were formed from borosilicate glass stock that was pulled to a suitable tip resistance (2-4 MΩ) by a Sutter P1000 (Sutter Instruments, Novato, CA). In experiments on K_v channels, the pipette solution contained (in mM): 120 KCl, 5 Na_2-ATP, 0.4 Na_2-GTP, 5 EGTA, 2.25 $CaCl_2$, 5 $MgCl_2$, 20 HEPES, adjusted to pH 7.4 with KOH. In experiments on Na_v channels, the pipette solution contained (in mM): 140

CsF, 10 NaCl, 5 EGTA and 10 HEPES, adjusted to pH 7.4 with CsOH. The osmolarity was approximately 290 mOsm.

Recording and Characterization of Muscle and Vascular Nociceptors

Whole cell patch clamp recordings were made with an Axopatch 200B (Molecular Devices, Sunnyvale, CA). Stimuli were controlled and records were captured with pClamp software and a Digidata 1322A. Series resistance (R_s) was compensated 60-75% with Axopatch compensation circuitry. Whole cell resistance and capacitance were determined by the Clampex software utility. Recorded currents were sampled at 10-20 kHz and filtered at 2 kHz (Bessel filter).

Once the whole cell mode was achieved, neurons were classified as type 5 (muscle) or type 8 (vascular) nociceptors using the method of Scroggs and Cooper (Cardenas et al., 1995; Petruska et al., 2000; 2002; see also Xu et al., 2010; Ono et al., 2010).

Isolation of $Na_v1.9$ Channel Currents

Following cell classification in Tyrode's solution, Na^+ currents were isolated in an external solution (Na_{iso}) containing (in mM): 20 or 70 NaCl, 120 or 70 TEA-Cl, 0.1 $CaCl_2$, 0.1 $CdCl_2$ and 10 HEPES, adjusted to pH 7.4 with TEA-OH. TTX (500 nM) was added prior to the days experiment. $Na_v1.9$ currents were recorded using the 70 mM Na_{iso}. The pipette solution contained 140 CsF, 10 NaCl, 5 EGTA and 10 HEPES, adjusted to pH 7.4 with CsOH.

Evocation and Characterization of $Na_v1.9$

From a V_h of –120 mV, cells were stepped from -80 to -20 mV in 5 mV steps (300 ms duration). Currents were leak corrected, on line, using the P/4 procedure module of Clampex 9.0. DEET or ETOH was applied, by close superfusion (~1 mm), for 2 minutes prior to testing. All Na_v characterizations were performed at room temperature (20°C). Series resistance was corrected 70-80%. Junction offsets were not corrected.

Peak currents of non-desensitizing $Na_v1.9$ were measured 250 msec from the start of the voltage step to avoid contamination by $Na_v1.8$. The slow desensitizing $Na_v1.8$ could appear at -20 mV but it would be fully desensitized within 50 msec of the voltage step. For voltage dependent activation, individual evoked peak currents were transformed into a conductance: $G=I_{peak}/(V_m-V_{rev})$, where I_{peak} was the test current, V_m the test command voltage, and V_{rev} was calculated from the Nernst equation to be 49.6 mV. The conductance was then normalized to the peak conductance (G_{max}) observed. The voltage dependence of activation was determined from a fit of the voltage-conductance measures to a Boltzmann function of the form: $G=G_{max}/(1+\exp((V_{.50}-V_m)/K))$, where $V_{.50}$ is the voltage at which G is half maximal, and K is a slope factor. Average currents were formed from the normalized peak currents observed over the active range (-65 to -40 mV).

Isolation of K_v7 Channel Currents

Following cell classification in a Tyrode's solution, K^+ currents were characterized in an external, K_{iso}, solution containing (in mM): 130 N-methyl-d-glucamine, 4 KCL, 4 $MgCl_2$, 0.2 $CaCl_2$, 1 $CsCl_2$, 2 4-aminopyridine, 10 glucose, 10 HEPES, adjusted to pH 7.4 with HCl. The

pipette solution contained (in mM): 120 KCl, 5 Na_2-ATP, 0.4 Na_2-GTP, 5 EGTA, 2.25 $CaCl_2$, 5 $MgCl_2$, 20 HEPES, adjusted to pH 7.4 with KOH.

Evocation and Characterization of K_v7 Current

A current subtraction method was used to isolate K_v7 mediated currents from other K^+ currents that were present as deactivation tail currents. The cell size normalized peak and average K_v7 current was assessed as a conductance to eliminate deactivation voltage confounding of the peak current. For the K_v7 deactivation protocol: a 1,000 msec step command to -20 mV was followed by a series of repolarizing 10 mV steps from -20 to -90 mV (1,000 ms; V_H = -60 mV) followed by a return step to -60 mV. A tail current could be measured during the repolarization steps. The K_v7 voltage deactivation protocol tests were conducted 3 minutes following application of the K^+ isolation solution containing ETOH or DEET. This was followed by application of the K_{iso} solution containing the K_v7 specific antagonist linopirdine (10 μM in ETOH; 3 min application). The K_v7 voltage deactivation protocol was reapplied. The linopirdine sensitive K_v7 current was isolated by subtraction.

The amplitude of the linopirdine sensitive tail current was measured from a point beginning 10 ms after the repolarizing voltage step (-30 to -90 mV) to the point 10 ms prior to the return step to -60 mV. The currents of individual cells were normalized by cell capacitance (pA/pF) and converted into a conductance (G) as described above, where V_{rev}=-86.5 mV. A mean G was computed over the range of functional deactivation steps (-40 to -70 mV) to obtain a mean normalized conductance. The peak conductance was determined by inspection.

Statistics on Behavior and Whole Cell Patch Studies

A repeated measures ANOVA was used to assess influence of GW chemical treatments on the development of pain signs (post-exposure weeks 5-12). In order to assess the persistence of pain behaviors, an additional analysis was conducted on the 4 week span proceeding euthanasia (post-exposure weeks 17-20 and/or 21-24, Group A and C only). Dependent measures included: 1) ambulation: movement distance (cm/15 min), average movement rate (cm/sec); and 2) rest duration (sec/15 min). The alpha level was set at .05. Although we had previously tested pressure pain, using the PAM device, this measure was dropped as it has never shown any sensitivity to the GW agent protocol.

On the advice of the study veterinarian, 1 rat was euthanized for health related issues (GRP A). To equalize the number of animals in each group, the corresponding rat (by date of entry), was excluded from each group. In addition, due to substantial variability inherent in rat behavior measures, the highest and lowest score of each group was excluded from the analyses. Therefore, for the analysis, the final number of rats per group was reduced.

Autonomic Nervous System Studies

Longitudinal measure of autonomic parameters and blood flow: To measure autonomic responses in blood flow in the hindpaw (plantar foot), rats were briefly anesthetized with isoflurane (10-15min as described below for terminal experiment) and placed in sternal recumbency. Using a noninvasive laser speckle contrast imager (LSCI PeriCamPSI, PeriMed, Inc.), blood flow

measurements were recorded for five minutes. The laser probe was positioned 14.9-15.1cm above the tissue. The LSCI sample rate was 53 samples/sec. LSCI technology uses a laser that illuminates the area measured with scattered light and produces a speckled pattern based on the red blood cell movement. This pattern is then captured by a built-in camera and digitized to produce an image with different corresponding colors that represent multiple interference patterns. Increased red blood cell movement results in a subsequent increased blurred speckled/interference pattern which then translates into increased blood flow. The analysis area on the hindpaw is set by the programmable software based on spatial landmarks and was used for all animals to validate inter-animal and within-animal comparisons. In longitudinal measures, after blood flow data was acquired, body core (rectal probe) and hindfoot (skin thermistors) temperatures were measured. Measurements were taken prior to chemical treatment (baseline), and every two weeks following treatment (2, 4, 6, 8, and 10 weeks) to examine chronic effects of GWI chemicals. Once blood flow measurements were completed, blood pressure and pulse rate were recorded using a computerized blood pressure system, Visitech BP2000 Series II Blood Pressure System, with the animal still anesthetized to prevent movement artifact. The blood pressure cuff was positioned at the base of the animal's tail and 3 consecutive measurements were recorded with a 10 second interval between pressure measurements. Averages of the three recordings were then used for data purposes. Anesthesia was then discontinued and animal returned to the cage for anesthetic recovery.

Surgical Preparation for Terminal in vivo Studies: In initial studies on exposed (all chemicals) and control rats 20-24 weeks after the end of exposure period, anesthesia was induced briefly with isoflurane from a calibrated vaporizer in a calibrated oxygen ventilator circuit interfaced with a rodent induction chamber with an approved scavenging system. Once the animal's movement

stopped, it was transferred to the procedure table and fitted with an isoflurane equipped inhalant nose-cone to maintain anesthesia. Baseline blood flow measurements of the hindpaws were recorded with the same methods as mentioned above. Prior to surgery, proper plane of anesthesia was ensured and indicated by loss of the withdrawal reflex, palpebral reflex, and pinna reflex. Using an esophageal probe, body temperature was maintained at 37ºC using a custom circulating water heating pad under the animal's core and hindlimbs. The trachea, common carotid artery, and jugular vein were intubated to assist with respiration, monitor blood pressure and heart rate, and provide IV access. Ventilation was monitored with an end tidal pCO2 monitor and artificial ventilation was used if necessary with a rodent ventilator supplied with oxygen. Baseline blood pressure was maintained at 75mmHg or above throughout the experiment. The animal was placed in the prone position and the left sciatic nerve was isolated within the ischiorectal fossa and surrounded by a silicon microelectrode cuff containing two silver stimulating electrodes.

Autonomic Measures in Terminal Experiments: After a short acting paralytic agent was administered to eliminate movement (atracurium besylate), LSCI measures of blood flow were obtained in the plantar hindfeet and surgically exposed surface of the distal half of the gastrocnemius muscles. Antidromic or reflex vasodilatation/vasoconstriction of the feet and muscle was evoked by a 3sec, 50Hz train burst stimulation (0.8ms pulse duration) of the (i) left distal sciatic nerve stump, (ii) left whole sciatic nerve stimulation, and (iii) proximal left sciatic nerve stump, were measured with LSCI.

Data Analysis: Blood flow recordings were analyzed using four regions of interest (ROI) that each outlined the left/right hindfeet and left/right heels. The ROI outlining the hindfeet incorporated the entire plantar surface excluding the digits; whereas ROI of the heels were standardized by measuring 1.6cm from the edge of the heel towards the distal midline. Mean blood

perfusion values (perfusion units) obtained during the five minute blood flow recordings were documented for statistical analysis. All longitudinal blood flow values were statistically analyzed using MINITAB Statistical Software (MINITAB release 7; Minitab, State College, PA) measuring normality and significant differences between mean perfusion units of exposed and control groups. Terminal experiment blood flow values of the left hindpaw and gastrocnemius muscle, following left distal sciatic nerve stimulation, were analyzed to determine significant differences between initial perfusion, measured at the end of the latent period of sciatic stimulation, and final (peak) perfusion of exposed and control rats.

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DEET Potentiates the Development and Persistence of Anticholinesterase Dependent Chronic Pain Signs in a Rat Model of Gulf War Illness Pain

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Key Words: Gulf War Illness; Chronic Pain; DEET; chlorpyrifos; pyridostigmine; $Na_v1.9$

Abstract

Exposure to DEET (N,N-diethyl-meta-toluamide) may have influenced the pattern of symptoms observed in soldiers with GWI (Gulf War Illness; Haley and Kurt, 1997). We examined how the addition of DEET (400 mg/kg; 50% topical) to an exposure protocol of permethrin (2.6 mg/kg; topical), chlorpyrifos (CP; 120 mg/kg), and pyridostigmine bromide (PB;13 mg/kg) altered the emergence and pattern of pain signs in an animal model of GWI pain (Nutter et al., 2015). Rats underwent behavioral testing before, during and after a 4 week exposure: 1) hindlimb pressure withdrawal threshold; 2) ambulation (movement distance and rate); and 3) resting duration. Additional studies were conducted to assess the influence of acute DEET (10-100 μM) on muscle and vascular nociceptor K_v7, K_{DR}, $Na_v1.8$ and $Na_v1.9$. We report that a 50% concentration of DEET enhanced the development and persistence of pain-signs. Rats exposed to all 4 compounds exhibited ambulation deficits that appeared 5-12 weeks post-exposure and persisted through weeks 21-24. Rats exposed to only three agents (CP or PB excluded), did not fully develop ambulation deficits. When PB was excluded, rats also developed rest duration pain signs, in addition to ambulation deficits. There was no evidence that physiological doses of DEET acutely modified nociceptor K_v7, K_{DR}, $Na_v1.8$ or $Na_v1.9$ activities. Nevertheless, DEET augmented protocols increased the activity of $Na_v1.9$ in muscle nociceptors harvested from chronically exposed rats. We concluded that DEET enhanced the development and persistence of pain behaviors, but the anticholinesterases CP and PB played a determinant role.

Introduction

Chronic pain is a common symptom of Gulf War Illness (GWI). More than 60% of US veterans of the 1991 Persian Gulf War developed a highly varied constellation of deep tissue pains that included headache, muscle, joint, and abdominal pain (Haley and Kurt, 1997; Blanchard et al., 2006; Stimpson et al., 2006; Thomas et al., 2006; Haley et al., 2013). While GWI typically developed soon after they returned from service in the Persian Gulf, a substantial portion of warfighters reported symptoms while still in theater (~25%; Kroenke et al., 1998). In the years that followed, the symptoms of GWI tended to remain the same or worsen over time (Hotopf et al., 2003). After more than 20 years of research, there is an emerging consensus that excessive exposure to insecticides and related agents contributed to the development of GWI symptoms (White et al., 2016). However, the relationship between particular exposure patterns and symptoms has remained elusive.

During their relatively brief deployment, the soldiers of ODS (Operation Desert Storm) were potentially exposed to 64 insecticides and repellants containing 37 distinct active ingredients (DoD Environmental Exposure Report: Pesticides, 2003; Binns et al., 2008; RAC, 2014). Our laboratory developed a rat model of Gulf War Illness pain as part of an effort to explore cellular

and molecular maladaptations associated with prolonged exposure to insecticides that were employed in ODS. Our early investigations focused on the role of three particular agents that possessed a unique potential to interact with membrane proteins expressed by deep tissue nociceptors. These agents included: 1) permethrin--- a type I pyrethroid that was supposed to be applied by soldiers to their uniforms every 4-5 days. Permethrin is a powerful Na_v (voltage activated sodium) channel deactivation inhibitor that lengthens action potential duration and thereby permits relatively massive amounts of Ca^{++} into intracellular space of nociceptors (Jiang et al., 2013); 2) chlorpyrifos---a powerful acetylcholinesterase (AChE) inhibitor with the potential to alter multiple cholinergic signaling mechanisms and pathways that are present in deep tissue nociceptors (Rau et al., 2005; Nutter et al., 2013; Cooper et al., 2016). Chlorpyrifos was used as an area spray/fogger and was also present in flea collars that soldiers obtained outside of their officially approved panel of agents (Binns et al., 2008); and 3) pyridostigmine bromide (PB)----an acetylcholinesterase inhibitor that soldiers were instructed to use as a prophylactic against potential nerve agent attack (Weinbroum, 2004; Newmark, 2005; Weissman and Raveh, 2011). PB was supposed to be self-administered by soldiers 3 times per day. Compliance with the prescribed doses and application frequencies of these chemicals was highly variable, and some agents were used excessively (Binns et al., 2008).

Exposing rats to various concentrations and durations of these three GW agents failed to produce a pattern of behavior changes consistent with chronic pain (Jiang et al., 2013; Nutter et al., 2013; Nutter and Cooper, 2014). We recently found that an intensified exposure to the AChE inhibitors (chlorpyrifos, PB) could produce pain-like behaviors that appeared and/or persisted up to 12 weeks following termination of exposure (Nutter et al., 2015). Although an 8 week protocol, utilizing an intermittent exposure pattern, and consisting of daily permethrin, chlorpyrifos (twice

per month; 7% duty cycle), and PB (14 days per month; 50% duty cycle) could not produce any lasting changes in rat activity levels or superficial pain measurements (Nutter et al., 2013; Nutter and Cooper, 2014), a doubling of the duty cycle of the anticholinesterases (chlorpyrifos to 14%; PB to 100%), did induce a delayed pain-like syndrome that emerged 9-12 weeks after exposure (Nutter et al., 2015). Using this anticholinesterase intensified protocol, pain-like behaviors were manifested as an increase in resting times and a decrease in free ranging ambulation. Patch clamp studies conducted on dorsal root ganglion neurons harvested from these same rats revealed the development of a variety of cellular and molecular maladaptations to muscarinic receptor (mAChR) signaling pathways and effectors in muscle nociceptors that were consistent with a chronic myalgia (Nutter et al., 2015; Cooper et al., 2016).

DEET (N,N-Diethyl-meta-toluamide) is an insect repellant that was commonly used by troops during their deployment (Binns et al., 2008). There is evidence that the application of DEET covaried with the development of pain symptoms in returning veterans (Haley and Kurt, 1997). DEET has no known direct interaction with the pain system, but has been shown to be a very weak anticholinesterase (Corbel et al., 2009; Wille et al., 2011; Swale et al., 2014). A recent publication demonstrated that high concentrations of DEET could inhibit both Na_v and K_v ion channel current amplitudes in rat cortical neurons (Swale et al., 2014; see also Corbel et al., 2009). Our laboratory has associated enhanced $Na_v1.9$ and decreased K_v7 activity with the appearance of pain-like signs in rats exposed to GW chemicals (Nutter and Cooper, 2014; Nutter et al., 2015; Cooper et al., 2016). In the experiments described below, we examined the impact of a DEET augmented exposure protocol on the development of pain signs in our rat model. Additional studies clarified the interaction of DEET with nociceptor ion channels implicated in the development of GWI pain.

Methods

Behavioral Studies

Subjects. Fifty (50) young adult male rats were used in the pesticide exposure studies (Sprague-Dawley; Envigo/Harlan). An additional 85 rats were used in physiology experiments. Rats entering the study weighed 90-110 grams. Terminal weights did not differ significantly in any pesticide exposure group (see Table 1). All animals were housed in American Association for Accreditation of Laboratory Animal Care approved quarters, and all procedures were reviewed and approved by the local Institutional Animal Care and Use Committee and ACURO (Animal Care and Use Review Office of the Army Medical Research and Materiel Command). Two rats developed health issues and were euthanized. After chemical exposures had ended, one rat manifested a rigidity of one hindlimb and the second rat developed a ventral midline tumor. There were no signs of acute pesticide toxicity typically associated with permethrin or chlorpyrifos during the execution of these studies.

Chronic Exposure Protocol. Over a period of 4 weeks, rats (n=50) were exposed to permethrin (2.6 mg/kg; mixture of 26.4% cis and 71.7% trans; Sigma Aldrich), chlorpyrifos (120 mg/kg; Sigma Aldrich), DEET (200 or 400 mg/kg; Sigma Aldrich) and pyridostigmine bromide (PB; 13 mg/kg; Sigma Aldrich). Permethrin, in ETOH, was applied every day to a shaved area of the back

(~1square inch) between the forelimbs. Chlorpyifos was administered by a subcutaneous injection (corn oil) once every 7 days. The dose of chlorpyrifos was intended to represent a net exposure to the potentially large and varied anticholinesterases that soldiers were exposed to in the Gulf theater (Binns et al., 2008). Chlorpyrifos was administered in a corn oil formulation that released the agent over a couple of days (Smith et al., 2009). DEET was administered topically in ethanol at one of two concentrations (25% or 50%). PB was administered daily by oral gavage (tap water) based upon a standard military dose that was adjusted to account for faster pharmacokinetics in rodents (Birtley et al 1966; Husain et al., 1968; Aquilonius et al., 1980; Breyer-Pfaff et al., 1985). Rats were weighed once per week throughout the studies and doses were adjusted accordingly. Control rats received only vehicle exposures over the identical time course.

Five distinct groups of rats (n=10) were formed (see Table 1). One group received all 4 agents (Group A). Three groups were exposed to DEET at 50% concentration (400 mg/kg; ETOH) while a fourth group received all 4 agents with DEET reduced to half concentration (Group HD; 200 mg/kg; 25% in ETOH). The latter group served as a positive control for DEET potentiation (Haley and Kurt, 1997). Two groups received only 3 agents: Group PB (PB excluded) and Group CP (chlorpyrifos excluded). Group C served as a vehicle control group. Agents were always administered in the same order. On the one day per week that rats were dosed with chlorpyrifos, the order was PB, chlorpyrifos, permethrin and then DEET. On the days in which chlorpyrifos was not administered, the order of administration was PB, permethrin and DEET. There was little indication that any combination of chemical exposures affected final body weight (Table 1).

Table 1

Group	Permethrin	Chlorpyrifos	PB	DEET	Body Weight
A	2.6*	120	13	400	489 ± 7.0#
HD	2.6	120	13	200	486 ± 5.2
CP	2.6	0	13	400	476 ± 6.07
PB	2.6	120	0	400	514 ±11.0
C	0	0	0	0	489 ± 8.0

*all doses in mg/kg # final weight in grams

Assessment of Pain Behaviors. Prior to entering the study, rats were acclimated to the behavioral procedures for 2 weeks. Pain assessments were conducted weekly throughout the entire dosing and post-dosing periods. A pressure-pain withdrawal threshold was measured using a computer monitored, hand held force transducer (PAM; Ugo Basile). Pressure was applied via a 5 mm diameter ball to the semitendinosus and biceps femoris muscles (left hind limb). During force application, the applied pressure was monitored and instantaneously displayed on a video screen. Video feedback enabled the rate of force application to be regulated by comparison to a standard curve. When the rat withdrew its limb, the force at withdrawal was automatically registered and stored. To complement pressure-pain testing, activity levels (movement distance, average movement rate, and rest time duration) were recorded automatically by infrared sensors in a modified activity box (15 min test period; Fusion Systems, AccuScan Instruments Inc.). The 35 by 40 cm test chamber was modified to prevent rearing behaviors. The chamber was cleaned after each 15 minute test period. Behavioral tests were conducted on both chemically exposed (permethrin, chlorpyrifos, DEET, PB) and vehicle treated (ETOH, corn oil, water) animals over

an identical time course. Rats were tested once per week on the behavioral tasks. PAM tests were conducted in 'blinded' conditions.

Electrophysiological Studies

Preparation of Cells. Dorsal root ganglion neurons (DRG) were harvested from young adult male rats (90-150 grams). Rats were anesthetized (Isoflurane) and rapidly euthanized by decapitation (Harvard Instruments). The spinal column was removed, bisected and the DRG were dissected free from T11 to S1. Ganglia were trimmed, cut into strips and digested in Tyrode's solution containing collagenase A (2 mg/ml; Roche Chemical) and Dispase II (5 mg/ml; Roche Chemical). A 15 ml centrifuge tube containing the dissected ganglia was placed in a heated, shaking water bath for 90 minutes at 35° C (EDVOTEK Digital Shaking Water Bath). Gentle trituration was then used to break up visible strips of ganglia. The dispersed neurons were then digested for an additional 45 minutes, and then spun at 500 RPM (30 sec). The supernatant was discarded. The remaining pellet was dispersed into 2 ml of Tyrode's, triturated and plated on 9, 35 mm, polylysine coated Petri dishes (Fluorodish). Plated neurons were bathed continuously in a Tyrode's solution, containing (in mM) 140 NaCl, 4 KCl, 2 $MgCl_2$, 2 $CaCl_2$, 10 glucose, and 10 HEPES, adjusted to pH 7.4 with NaOH. All electrophysiological studies were conducted at room temperature (20 °C) within 10 hours of plating. Only one cell was used per Petri dish. Electrodes were formed from borosilicate glass stock that was pulled to a suitable tip resistance (2-4 MΩ) by a Sutter P1000 (Sutter Instruments, Novato, CA). In experiments on K_v channels, the pipette solution contained (in mM): 120 KCl, 5 Na_2-ATP, 0.4 Na_2-GTP, 5 EGTA, 2.25 $CaCl_2$, 5 $MgCl_2$, 20 HEPES, adjusted

to pH 7.4 with KOH. In experiments on Na_v channels, the pipette solution contained (in mM): 140 CsF, 10 NaCl, 5 EGTA and 10 HEPES, adjusted to pH 7.4 with CsOH. The osmolarity was approximately 290 mOsm.

Recording and Characterization of Muscle and Vascular Nociceptors. Whole cell patch clamp recordings were made with an Axopatch 200B (Molecular Devices, Sunnyvale, CA). Stimuli were controlled and records were captured with pClamp software and a Digidata 1322A. Series resistance (R_s) was compensated 60-75% with Axopatch compensation circuitry. Whole cell resistance and capacitance were determined by the Clampex software utility. Recorded currents were sampled at 10-20 kHz and filtered at 2 kHz (Bessel filter).

Once the whole cell mode was achieved, neurons were classified as type 5 (muscle) or type 8 (vascular) nociceptors using the method of Scroggs and Cooper (Cardenas et al., 1995; Petruska et al., 2000; 2002; see also Xu et al., 2010; Ono et al., 2010). Categorization of cells by 'current signatures' permits relatively simple identification of distinct cell groups with uniform physiological properties and anatomical targets. Categorization procedures have evolved since they were first established by the Scroggs laboratory and subsequently expanded by our laboratory. Using 3 voltage characterization protocols (CP1, CP2 and CP3), we classified small and medium sized neurons as type 5 muscle or type 8 vascular nociceptors. The physiological signature of type 5 nociceptors used in this study included small I_H (1.18 ± 0.22 pA/pF; CP1), a high threshold I_A (0 mV; CP2) that exhibited a prolonged settling time (55.4 ± 0.87 msec) and a high threshold (> -20 mV; CP3), broad (5.47 ± 0.20 msec at baseline; 0 mV test) Na^+ current. Type 5 nociceptors were found in both the small and medium sized cell pool (30-45 μM diameter; 80.5 ± 2.92 pF). The physiological signature of type 8 nociceptors included small I_H (1.10 ± 0.19 pA/pF; CP1), an I_A

threshold of -20 mV with prolonged I_A settling time (57.6 ± 1.60 msec; CP2), and a high threshold (> -20 mV; CP3), broad (4.61 ± 0.23 msec at baseline; 0 mV test) Na^+ current. Type 8 nociceptors were found among the medium sized cell population (35-45 μM diameter; 78.5 ± 2.68 pF). The main distinguishing feature between type 5 and type 8 cells was the 20 mV difference in the threshold of I_A. Their signatures are very different from other medium sized neurons encountered in DRG recordings. Those neurons typically feature combinations of large I_H, low threshold, fast settling I_A and low threshold Na^+ currents with fast kinetics (Petruska et al., 2000; 2002). Cells not fitting the classification criteria of type 5 or 8 were discarded. Anatomical targets of type 5 and type 8 neurons were determined by a series of anatomic tracing experiments (Jiang et al., 2006; Rau et al., 2007; Rau et al., 2014; Cooper et al., 2014). Type 5 and type 8 nociceptors are capsaicin/heat sensitive and co-express vasoactive neuropeptides (substance P and CGRP; Petruska et al., 2000, 2002; Rau et al., 2007).

Isolation of $Na_v1.8$ and $Na_v1.9$ Channel Currents. Following cell classification in Tyrode's solution, Na^+ currents were isolated in an external solution (Na_{iso}) containing (in mM): 20 or 70 NaCl, 120 or 70 TEA-Cl, 0.1 $CaCl_2$, 0.1 $CdCl_2$ and 10 HEPES, adjusted to pH 7.4 with TEA-OH. TTX (500 nM) was added prior to the days experiment. $Na_v1.9$ currents were recorded using the 70 mM Na_{iso} solution while $Na_v1.8$ currents were recorded using the 20 mM Na_{iso} solution. The pipette solution contained 140 CsF, 10 NaCl, 5 EGTA and 10 HEPES, adjusted to pH 7.4 with CsOH.

Evocation and Characterization of $Na_v1.9$. From a V_h of –120 mV, cells were stepped from -80 to -20 mV in 5 mV steps (300 ms duration). Currents were leak corrected, on line, using the P/4 procedure module of Clampex 9.0. DEET or ETOH was applied, by close superfusion (~1 mm), for 2 minutes prior to testing. All Na_v characterizations were performed at room temperature (20°C). Series resistance was corrected 70-80%. Junction offsets were not corrected.

Peak currents of non-desensitizing $Na_v1.9$ were measured 250 msec from the start of the voltage step to avoid contamination by $Na_v1.8$. The slow desensitizing $Na_v1.8$ could appear at -20 mV but it would be fully desensitized within 50 msec of the voltage step. For voltage dependent activation, individual evoked peak currents were transformed into a conductance: $G=I_{peak}/(V_m-V_{rev})$, where I_{peak} was the test current, V_m the test command voltage, and V_{rev} was calculated from the Nernst equation to be 49.6 mV. The conductance was then normalized to the peak conductance (G_{max}) observed. The voltage dependence of activation was determined from a fit of the voltage-conductance measures to a Boltzmann function of the form: $G=G_{max}/(1+\exp((V_{.50}-V_m)/K))$, where $V_{.50}$ is the voltage at which G is half maximal, and K is a slope factor. Average currents were formed from the normalized peak currents observed over the active range (-65 to -40 mV).

Evocation and Characterization of $Na_v1.8$. Currents were isolated in the Na_{iso} solution as described above. Following a conditioning pulse to -70 mV (1,000 msec; V_H=-60 mV) a strongly depolarizing step to 0 mV (60 msec), evoked a large amplitude slowly desensitizing inward current. Currents were leak corrected, on line, using the P/4 procedure module of Clampex 9.0. After a stable baseline current was achieved, DEET or ETOH was applied for 9 minutes by close superfusion. Time dependent changes to the peak $Na_v1.8$ current were examined over a period of

7 minutes (2 minutes following application of DEET/ETOH; 15 sec intertrial interval). Peak currents were normalized to cell size (pF). The series resistance was corrected 60-70%. Junction offsets were not corrected. The peak $Na_v1.8$ current was measured from the peak current to a point (2500 msec) following the voltage step to 0 mV.

Isolation of K_{DR} and K_v7 Channel Currents. Following cell classification in a Tyrode's solution, K^+ currents were characterized in an external, K_{iso}, solution containing (in mM): 130 N-methyl-d-glucamine, 4 KCL, 4 $MgCl_2$, 0.2 $CaCl_2$, 1 $CsCl_2$, 2, 4-aminopyridine, 10 glucose, 10 HEPES, adjusted to pH 7.4 with HCl. The pipette solution contained (in mM): 120 KCl, 5 Na_2-ATP, 0.4 Na_2-GTP, 5 EGTA, 2.25 $CaCl_2$, 5 $MgCl_2$, 20 HEPES, adjusted to pH 7.4 with KOH.

Evocation and Characterization of K_v7 Current. A current subtraction method was used to isolate K_v7 mediated currents from other K^+ currents that were present as deactivation tail currents. The cell size normalized peak and average K_v7 current was assessed as a conductance to eliminate deactivation voltage confounding of the peak current. For the K_v7 deactivation protocol: a 1,000 msec step command to -20 mV was followed by a series of repolarizing 10 mV steps from -20 to -90 mV (1,000 ms; V_H = -60 mV) followed by a return step to -60 mV. A tail current could be measured during the repolarization steps. The K_v7 voltage deactivation protocol tests were conducted 3 minutes following application of the K^+ isolation solution containing ETOH or DEET. This was followed by application of the K_{iso} solution containing the K_v7 specific antagonist linopirdine (10 μM in ETOH; 3 min application). The K_v7 voltage deactivation protocol was reapplied. The linopirdine sensitive K_v7 current was isolated by subtraction.

The amplitude of the linopirdine sensitive tail current was measured from a point beginning 10 ms after the repolarizing voltage step (-30 to -90 mV) to the point 10 ms prior to the return step to -60 mV. The currents of individual cells were normalized by cell capacitance (pA/pF) and converted into a conductance (G) as described above, where V_{rev}=-86.5 mV. A mean G was computed over the range of functional deactivation steps (-30 to -70 mV) to obtain a mean normalized conductance. The peak conductance was determined by inspection.

Evocation and Characterization of K_{DR} Currents. For the purpose of this study, the K_{DR} current was defined as the total 4-AP insensitive K^+ current following removal of the K_v7 component with linopirdine. The voltage dependent activation of the total K_{DR} current, was assessed, as a tail current, after application of the K_v7 inhibitor linopirdine (10 μM; 8 min). From a holding potential of -60 mV, a 2,000 msec conditioning pulse (-100 mV) was followed by 12 consecutive command steps from -80 to 20 mV (10 mV increments; 500 msec duration). The amplitude of the tail current at -60 mV was measured from the peak relative to the baseline current recorded 2,500 msec after repolarization. For each recorded neuron, the amplitude of tail current was normalized to the peak evoked current and then plotted against the activation voltage to obtain a current-voltage relationship. A Boltzmann function was fit and a $V_{.50}$ determined for each individual cell. The voltage dependence of activation was determined from a fit of the voltage-current measures to a Boltzmann function of the form: $I=I_{max}/(1+\exp((V_{.50}-V_m)/K))$, where $V_{.50}$ is the voltage at which the current (I) is half maximal, and K is a slope factor.

To assess average amplitude, the K_{DR} tail currents, at each voltage, were normalized for cell size (current amplitude (pA) divided by the cell size parameter (pF)). These normalized

amplitudes were averaged across functional activation voltages (-60 to 0 mV) to obtain a mean current amplitude.

Statistics

A repeated measures ANOVA was used to assess influence of GW chemical treatments on the development of pain signs (post-exposure weeks 5-12). In order to assess the persistence of pain behaviors, an additional analysis was conducted on the 4 week span proceeding euthanasia (post-exposure weeks 17-20 and/or 21-24, Group A and C only). Dependent measures included: 1) muscle pain threshold (PAM; grams); 2) ambulation: movement distance (cm/15 min), average movement rate (cm/sec); and 3) rest duration (sec/15 min). The alpha level was set at .05. As noted above, 2 rats were euthanized for health related issues (one rat from Group A and one rat from Group HD). Both were terminated on the advice of the study veterinarian. To equalize the number of animals in each group, the corresponding rat (by date of entry), was excluded from each group. In order to adjust for the high variance, present in rat behavioral data, the means of movement and resting scores were trimmed (highest and lowest scores) prior to construction of plots and performance of analyses (Lix and Keselman, 1998; Wilcox et al., 2000; Mudholkar et al., 2013).

To determine the influence of DEET on physiology measures, Student's t-tests were used to contrast normalized amplitude, conductance and/or $V_{.50}$ of $Na_v1.8$, $Na_v1.9$, K_{DR} and K_v7 in DEET and vehicle (ETOH) treated cells. The alpha level was set at .05.

Results

Behavior

After a period of acclimation and baseline behavioral testing, rats were divided into 5 groups (n=50). One group (Group A) was treated with all four GWI chemicals for 4 weeks (permethrin 2.6 mg/kg, chlorpyrifos 120 mg/kg, PB 13 mg/kg, DEET, 400 mg/kg; 50% in ETOH). A positive control for the influence of DEET (Group HD) received all 4 agents, but DEET was administered at half the concentration (200 mg/kg; 25% in ETOH). Studies of GW veterans had indicated that skin application of high (75%), but not low concentrations (25%) of DEET, posed a risk factor for chronic pain (Haley and Kurt, 1997). Our preliminary studies indicated that a 50% level was sufficient to produce enduring pain-like signs. The remaining 2 groups received combinations of 3 agents where either chlorpyrifos (Group CP) or PB (Group PB) was not included in the dosing routine. A final group served as a vehicle treated control (Group C; corn oil, ethanol, water). Details of the dosing schedule are provided in 'Methods' and Table 1. Behavior assessment tests were conducted on all rats once per week for 26 weeks (Group PB, Group CP, Group HD) or 30 weeks (Group C, Group A). Tests included muscle pressure withdrawal threshold (PAM; left semitendinosus) and 3 open field activity measures: movement distance (cm), average movement rate (cm/sec), and rest time duration (sec). All PAM measures were carried out under blinded test conditions. Activity measures were assessed over a period of 15 minutes, in a 35 x 40 cm Perspex test chamber where movements were monitored and quantified by an automated infrared detection system (AccuScan).

After the 4 week exposure period had ended, we conducted a repeated measures ANOVA on 8 and 4 week blocks that extended from post-exposure week 5 through 24. We had previously

reported that rats exposed to the same concentrations of permethrin, chlorpyrifos and PB, but in the absence of DEET, exhibited only paradoxical shifts in movement rate and transient elevations of rest time scores during a 5 to 12 post-exposure assessment (Nutter et al., 2015). In studies of DEET augmented protocols below, we focused on this 5-12 week post-exposure time period as well as upon test periods extending up to 24 weeks after exposure.

With the addition of DEET (50% in ETOH) to the same 4 week exposure protocol, a consistent pattern of pain-like signs emerged and persisted for up to 6 months (figure 1A, B, C). Both ambulation measures, movement distance and movement rate, were significantly reduced in the immediate weeks following exposure (weeks 5-12; $F=19.47$, $p<.001$ and $F=27.71$; $p<.001$, respectively). Although the depression of movement distance returned to normal levels by the final assessment period, (weeks 21-24), the slowing of movement rate, due to GWI agents, was still significant 21-24 weeks post-exposure (figure 1B).

While there was a substantial augmentation of ambulation related pain signs when DEET was part of the exposure protocol, there was no evidence that rest durations were similarly affected (figure 1C). We previously reported persistent rest time increases with 8 week exposures to the 3 GW chemicals (chlorpyrifos, permethrin and PB; Nutter et al., 2015; Cooper et al., 2016), but not with a 4 week exposure to the same agents at identical dosages. As in all our investigations, we also failed to find that these GW agents produced any change in the semitendinosus muscle pressure-pain withdrawal test (PAM; data not shown; Nutter et al., 2014; Nutter et al., 2015; Cooper et al., 2016). GWI veterans do not report mechanical allodynia to superficial stimuli.

As shown in figure 1, exposure to the 4 agents produced a pattern of activity suppression *during* the 4 week exposure period that was observed in nearly all experiments below (figures 2-

4). Movement distance and average rate were substantially suppressed while resting duration was increased in the presence of DEET, chlorpyrifos, permethrin and PB (F=62.67, F=64.16 and F=10.55 respectively; figure 1A, B and C, 'Exposure'). However, the capacity of a given agent or agents to suppress activity scores during exposure was not necessarily related to whether post-exposure pain-like behaviors would occur or persist 5 to 6 months after exposures had ended (see below).

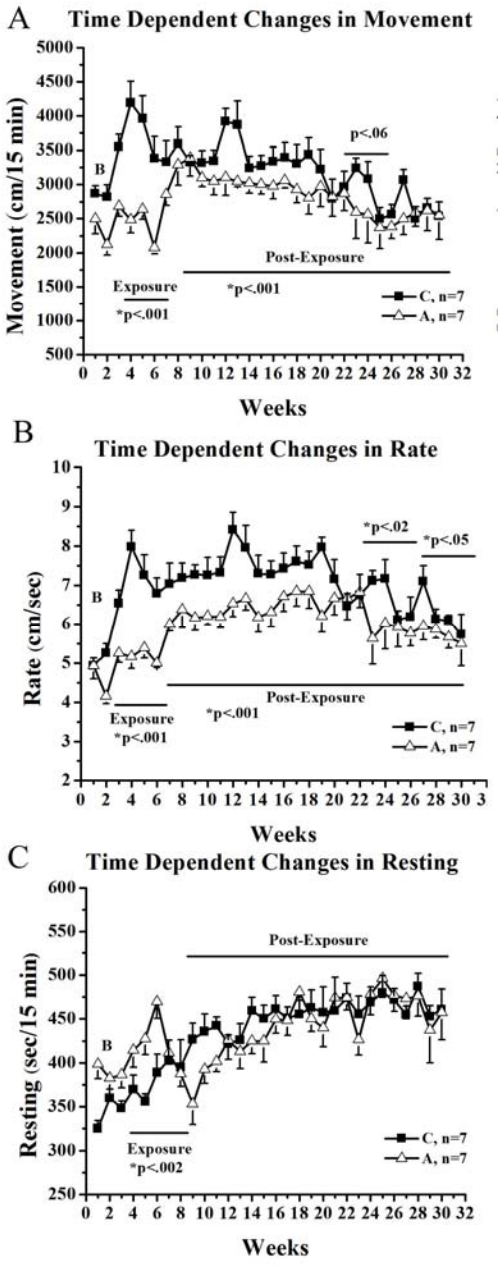

Figure 1. A DEET Augmented Exposure Protocol Produced Long Lasting Pain-Like Behaviors. A) Movement distance was significantly decreased at 5-12 weeks post exposure ($F=19.47$; $p<.001$). Movement distance pain signs approached significance 17-20 weeks post exposure ($F=3.72$; $p<.06$), but faded in the final month to testing (weeks 21-24). **B)** Average movement rate was significantly decreased 5-12 weeks post exposure ($F=22.71$; $p<.001$). Significant rate decreases were maintained out to weeks 21-24 ($F=4.00$; $p<.05$). **C)** Resting duration was unchanged during all post exposure test periods. Tests were not conducted on any measure 1-4 weeks post exposure. B: baseline testing; A: DEET, chlorpyrifos, PB, permethrin. C/Vehicle: (ethanol, corn oil, ethanol, water). *significantly different by ANOVA.

Consistent with reports on symptomatic veterans, high concentrations of DEET were associated with the development GWI pain-like behaviors in rats (Haley and Kurt, 1997). When the concentration of DEET was reduced to 25% (Group HD; positive control), neither ambulation nor resting measures were shifted in a manner consistent with pain (figure 2A, B and C). Instead, movement distance and resting scores were paradoxically increased (movement distance) or decreased (resting duration), relative to vehicle treated groups, during the final 4 weeks of testing (post-exposure weeks 17-20). When Group HD was contrasted with Group A (50% DEET), significant pain signs were evident in Group A at both the early phase (5-12 weeks post-exposure) and the late phase of assessment (weeks 17-20; figure 2D, E and F). Interestingly, although no pain-like signs developed with the HD group, we still observed highly significant decreases in ambulation (movement distance and rate), as well as increased rest durations during the exposure period (figure 2A, B and C; F=48.42, F=30.45 and F=5.35, respectively; 'Exposure').

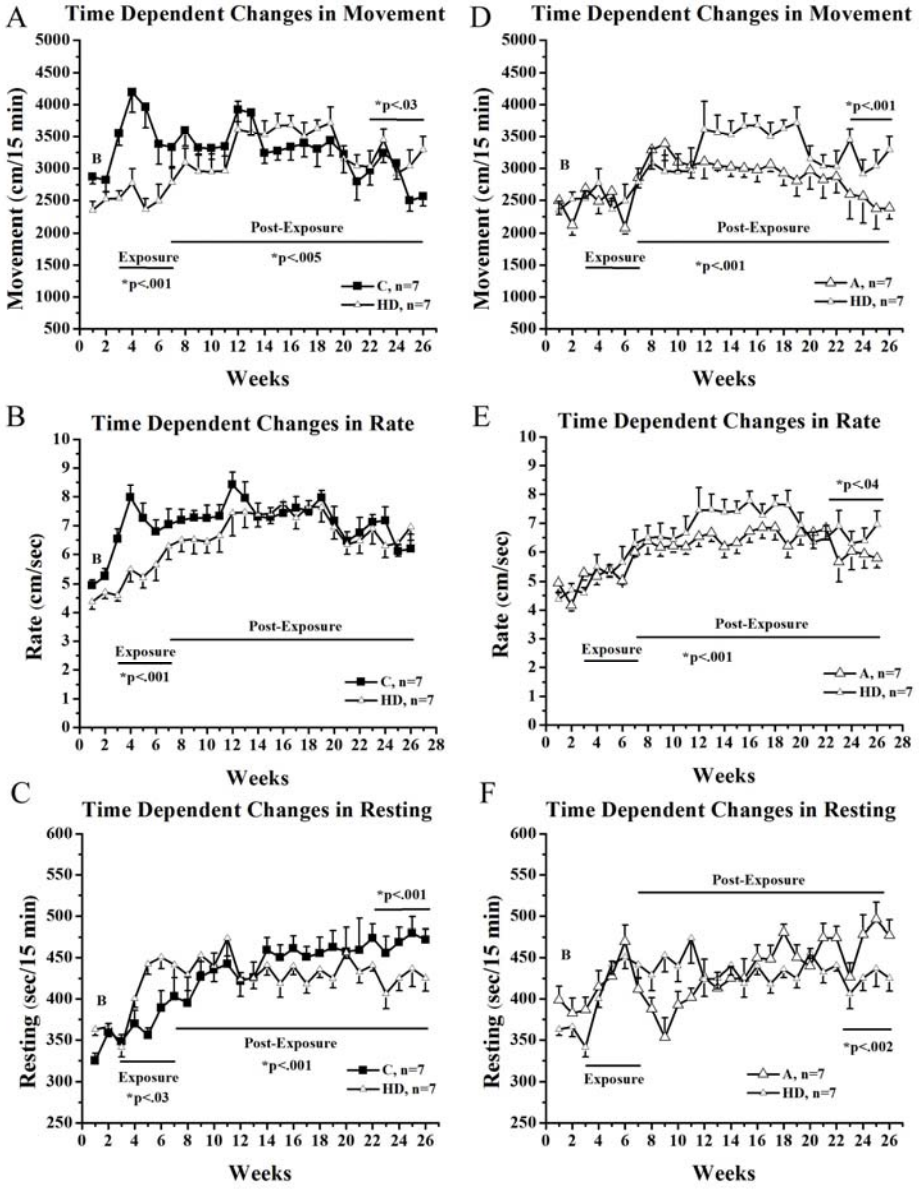

Figure 2. Pain-Like Behaviors Did Not Develop Nor Persist When DEET Concentration was Halved. A) Relative to control animals, movement distance was paradoxically increased in during post-exposure weeks 17-20 (F=5.50). **B)** Average movement rate was unchanged compared to vehicle exposed controls. **C)** Resting duration was paradoxically decreased relative to control rats in Group HD (weeks 17-20; F=19.51). There were no indications of pain signs during the early post-exposure period in any measure (weeks 5-12). **D and E)** When compared to Group A (50% DEET), the reduction of DEET concentration to 25% produced movement distance and rate scores that were significantly higher (more normal) during the last observation period (post-exposure weeks 17-20; F=12.4, p<.001 and F= 17.2, p<.001, distance and rate respectively). Pain behaviors appeared during the early testing phase in Group A (F=16.7 and F=16.1; p<.001, movement distance and rate, respectively). **F)** At week 17-20, resting was significantly reduced in the 25% DEET concentration group (F=11.24; p<.002), but there were no accompanying shifts during the early post-exposure phase (weeks 5-12). B: baseline testing; A: DEET (400 mg/kg; 50%), PB, chlorpyrifos, permethrin; C/Vehicle: (ethanol, corn oil, ethanol, water); HD: DEET (200 mg/kg; 25%), chlorpyrifos, PB, permethrin; *significantly different by ANOVA.

Conditions for the Development of GWI Pain Signs in Rats

Given that DEET substantially enhanced the development and persistence of pain-like behaviors, we conducted a series of experiments in order to determine if the development of these behaviors would appear and persist if the anticholinesterases were excluded from the dosing protocol. Our previous report had shown that doubling the anticholinesterase duty cycle had been critical to the development of pain signs in the 8 week protocol. In order to assess the role of AChE inhibitors, we contrasted Groups A and C with groups in which either chlorpyrifos (Group CP) or PB (Group PB) was excluded from the exposure regimen.

The Contribution of Chlorpyrifos. CP was originally included in the exposure set in order to represent the large variety of anticholinesterase insecticides that warfighters were exposed to during their deployment (Binns et al., 2008). Statistical analyses comparing vehicle treated rats (Group C) and rats exposed to all agents, except chlorpyrifos (Group CP), confirmed that CP was an essential component of those GW chemicals that induced persistent pain-like behaviors. When CP was omitted from the exposure protocol (Group CP), ambulation deficits were not sustained at post-exposure weeks 17-20 (figure 3A and B). Although transient shifts in movement rates were present through post exposure weeks 5-12 (figure 3B), movement distance scores also failed to emerge during weeks 5-12 in the absence of CP. When comparisons were made between rats exposed to all 4 agents (Group A) and Group CP, rats that were not exposed to CP exhibited significantly improved ambulation during all phases of the study (figure 3D and E).

It also appeared that CP contributed significantly to the suppression of movement distance and, to a lesser extent, movement rate that occurred during the 4 week exposure period (figure 3;

'Exposure'). As noted above, rats receiving all 4 GWI chemicals typically manifested a substantial reduction of ambulation scores (and elevation of rest times) during the 4 week exposure session (figure 1A, B and C; 'Exposure'). In the absence of CP, the suppression of both movement distance and rate were significantly relieved relative to Group A (figure 3D and E; 'Exposure'; F=21.99 and F= 42.11). Nonetheless, some weaker suppression of movement rate and induction of resting could still be detected relative to Group C (figure 3B; 'Exposure'; F=12.12).

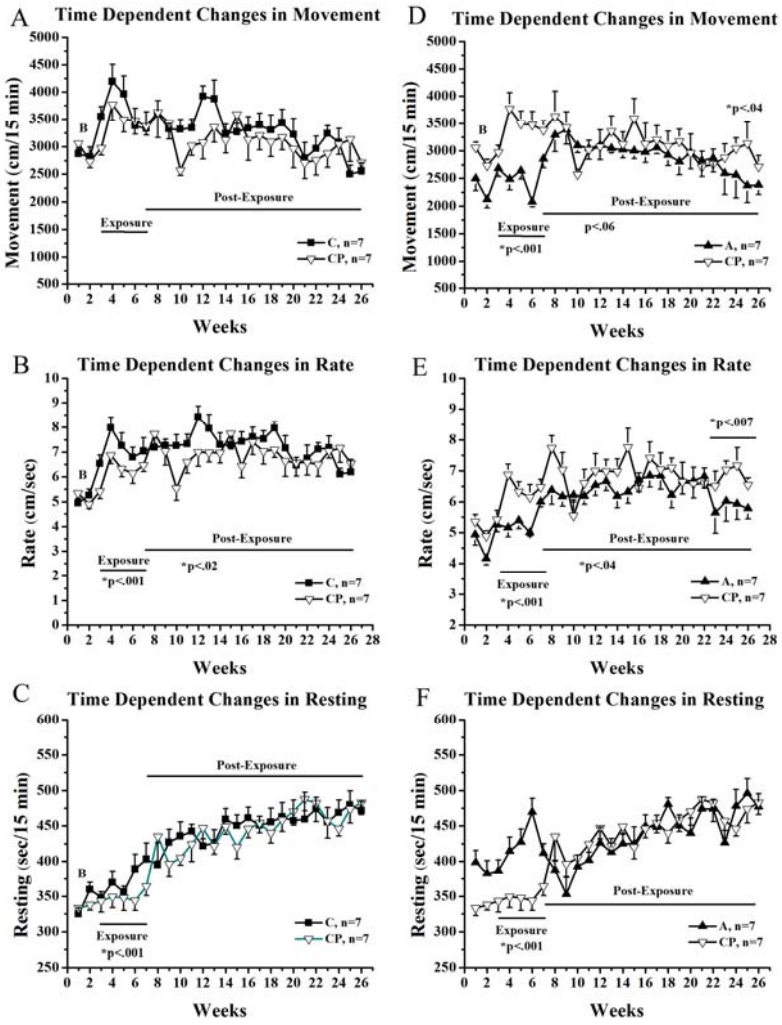

Figure 3. Excluding the AChE Inhibitor, Chlorpyrifos, from the Exposure Protocol Prevented Development of Persistent Pain Behaviors. A) The omission of CP prevented the suppression of movement (distance) by GW chemicals. **B)** Average movement rate was still significantly reduced in the early post-exposure phase in the absence of CP (weeks 5-12; F=6.11). Persistent changes in movement rate did not develop in the absence of CP (post-weeks 17-20). **C)** Rest durations remained unaffected when CP was absent. **D and E)** In the absence of chlorpyrifos, movement rate scores were shifted significantly towards vehicle exposure levels relative to groups that were exposed to all 4 agents. Significant rescue was observed over post-exposure weeks 5-12 (rate: F=4.37) and 17-20 (F=4.67 and F=7.84, movement distance and rate respectively). **F)** The exclusion of CP shifted resting scores toward vehicle levels only during the period of exposure (F=42.11). No other shifts were observed relative to Group A rats. B: baseline testing; A: DEET, chlorpyrifos, PB, permethrin; C: (ethanol, corn oil, ethanol, water); CP: DEET, PB, permethrin. *significantly different by ANOVA.

The Contribution of Pyridostigmine Bromide. Exclusion of PB from the chemical exposure protocol substantially altered the development of pain-like behaviors. Like the other anticholinesterase in the exposure set (CP), the inclusion of PB in the 4 week exposure period was necessary for the development of pain signs of movement distance and rate decreases that persisted into weeks 17-20; it was also required for development of these pain behaviors during post-exposure weeks 5-12 (Group PB vs Group C; figure 4A and B). Moreover, in the absence of PB, ambulation scores were significantly shifted towards normal levels relative to animals exposed to all 4 compounds (Group PB vs Group A; figure 4D and E).

Unlike CP, the absence of PB from the exposure protocol did not affect movement scores *during* the exposure protocol (figure 4A, B and C, 'Exposure'), and more importantly proved to be permissive for the development and persistence of resting pain signs over the course of post-exposure testing. Rats that did not receive PB treatment developed significant and substantial increases in resting behaviors over weeks 5-12 (Group C vs Group PB). These behaviors persisted into weeks 17-20 (figure 4C). Moreover, when Group PB resting times were compared to Group A, scores were not shifted toward normal levels. Instead, resting pain signs were further increased relative to the group that received all 4 agents (figure 4E). Therefore, the contribution of PB was to exert a protective influence against the development of pain associated with increased resting. Given that PB was prescribed to soldiers as a prophylactic against nerve agent anticholinesterases (i.e., Soman/Sarin), it is not surprising that such a finding could emerge. However, it is not a simple matter to reconcile this finding with the influence on movement distance and rate, in which PB contributed to impairments over the entire post-treatment period.

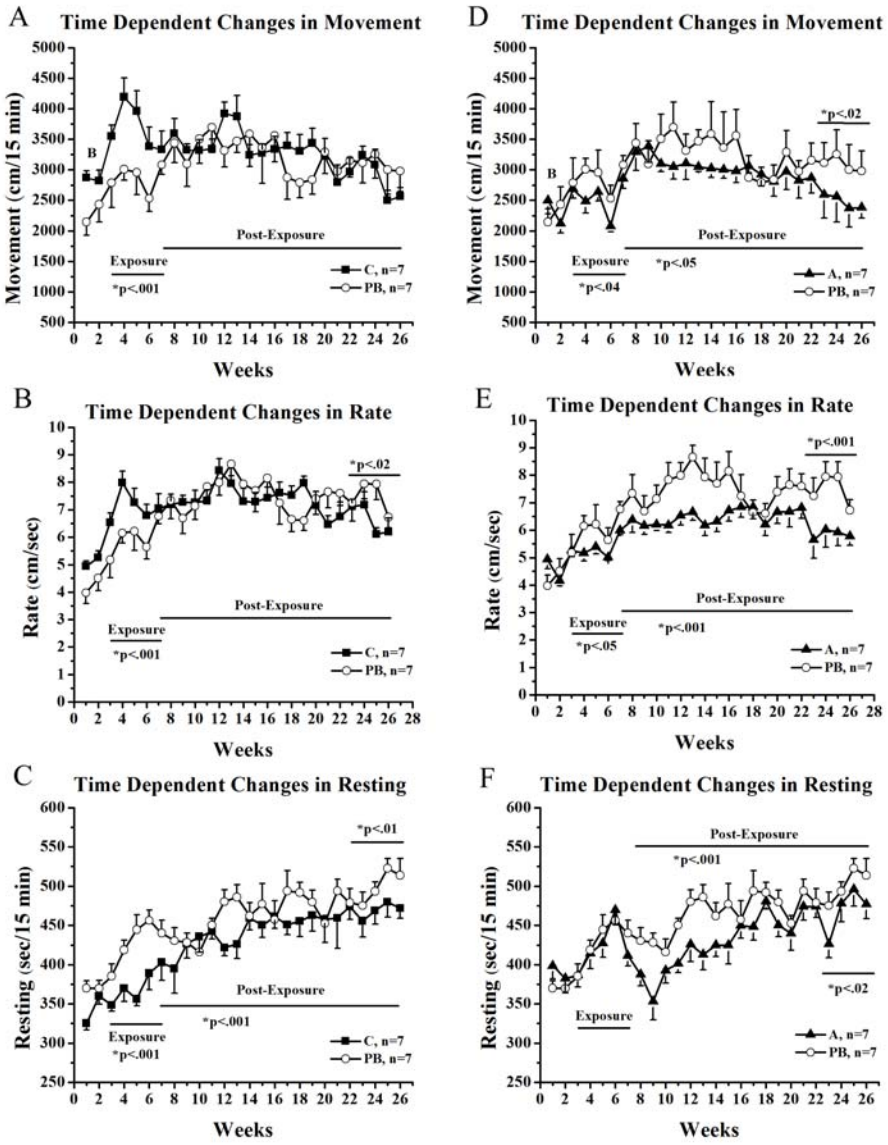

Figure 4. Excluding Pyridostigmine Bromide from the Exposure Protocol Differentially Contributed to the Development and Persistence of Pain Behaviors. A) Movement distance was unaffected in the absence of PB. **B)** Except for a paradoxical increase in weeks 17-20 (F=5.05), the average movement rate was also unaffected by GWI chemicals when PB was excluded from the exposure set. **C)** In the absence of PB, significant increases in rest duration scores emerged during the early post-exposure phase (weeks 5-12; F=11.60) and persisted into the final month of measurement (post-weeks 17-20; F=7.27). **D and E)** In the absence of PB, final movement distance and rate scores were shifted significantly towards vehicle exposure levels relative to groups that were exposed to all 4 agents (weeks 17-20: F=6.04 and F=17.34, movement distance and rate respectively). Movement distance and rate scores were also rescued during the early post-exposure phase (weeks 5-12: F= 4.00 and F=23.69, respectively). **F)** The exclusion of PB accentuated the influence of the remaining 3 GW chemical on rest durations during both post-exposure assessment periods (weeks 5-12: F =18.49; weeks 17-20; F=6.19). B: baseline testing; A: DEET, chlorpyrifos, PB, permethrin; C: (ethanol, corn oil, ethanol, water); PB: DEET, chlorpyrifos, permethrin. *significantly different by ANOVA.

The Influence of DEET on K_v and Na_v Ion Channel Physiology

Our previous studies indicated that an 8 week exposure to 3 GWI chemicals (chlorpyrifos, permethrin and PB) increased rest time durations and lowered movement distance scores 9-12 weeks post-exposure. Patch clamp physiology performed on cells harvested from those rats revealed that muscle nociceptors exhibited decreased net activity of K_v7 and other K_{DR} ion channels (Nutter et al., 2015). We further demonstrated that muscle nociceptors manifested a unique action potential burst discharge in response to activation of muscarinic receptors (mAChR); and that these action potential bursts were modulated by K_v7 and potentiated in rats exposed to the GWI chemicals (Nutter et al., 2014; Nutter et al., 2015). The low voltage activated K^+ channel, K_v7, is known to be important for governing neuronal excitability (Brown and Passmore, 2009).

As the inclusion of DEET in the exposure protocol intensified and shifted the pattern of behavioral outcomes, we hypothesized that DEET might also influence the activity of K_v (and other ion channels) channels that have been associated with the development of pain-signs in rats (K_{DR}). It was recently shown that DEET (~100 μM) significantly diminished the amplitude of the K_{DR} in cultured rat cortical neurons (Swale et al., 2014). Therefore, we initiated a series of studies which examined the influences of DEET on nociceptor K_v7 ion channels and the residual K_{DR} (that K_{DR} remaining after K_v7 and 4-AP sensitive channels were removed). Studies were focused on muscle nociceptors as these were the class of neurons that had been shown to be modified by GW agent protocols that produced persistent pain-like behaviors in our rat model (Nutter et al., 2015; Cooper et al., 2016).

The Influence of DEET on Nociceptor K_v7 and K_{DR}. Young adult male rats served as subjects. Cells were harvested and plated on the morning of the experiment, and discarded afterwards. Recordings began approximately 2 hours after plating. Neurons identified as muscle or vascular nociceptors were exposed to DEET (10 or 50 μM). K_v channels were isolated from other voltage activated currents using a K_{iso} solution described in 'Methods'. In control cases, equal volumes of vehicle (ethanol/ETOH) were substituted for DEET. In the case of K_{DR}, Boltzmann functions were used to describe the voltage dependent data. Individual cases in which Boltzmann functions could not be fit to K_{DR} currents were excluded from the analysis.

As shown in figure 5, we were unable to detect any influence of DEET (10-50 μM) on voltage dependence or amplitude of either K_v7 or K_{DR} ion channels expressed in muscle nociceptors. There were no influences of DEET on average (figure 5A) or peak conductance (not shown) of K_v7. Nor were changes observed in the calculated $V_{.50}$ or average currents evoked from K_{DR} channels (figure 5B and C). Accordingly, the amplification of behavioral pain signs exhibited 5-24 weeks after DEET exposure, could not be attributed to a direct influence of DEET on K_v channels during the exposure.

The Influence of DEET on Nociceptor Na_v. Acute exposure to pyrethroid insecticides, such as permethrin, have powerful influences on the properties of $TTX_{sensitive}$ ($Na_v1.6$, $Na_v1.7$) and $TTX_{insensitive}$ ($Na_v1.8$) ion channels expressed in DRG (Ginsburg and Narahashi, 1993; Tatebayashi and Narahashi, 1994; Tabarean and Narahashi, 1998; Tabarean and Narahashi, 2001; Jiang et al., 2013). $Na_v1.8$ is expressed in a high proportion of nociceptors and is the principle Na_v that forms action potentials in these neurons (Djouhri et al., 2003; Jiang and Cooper, 2011). Prolonged

exposure to permethrin, chlorpyrifos and PB changes inactivation characteristics of $Na_v1.8$ and prolongs action potential duration (Nutter et al., 2013; Nutter and Cooper, 2014; Nutter et al., 2015). Given that high concentrations of DEET (>500 μM) were recently shown to reduce the amplitude of mixed cortical Na_v, we examined whether such effects could be identified in DRG nociceptors at levels which might be physiologically significant (Swale et al., 2014).

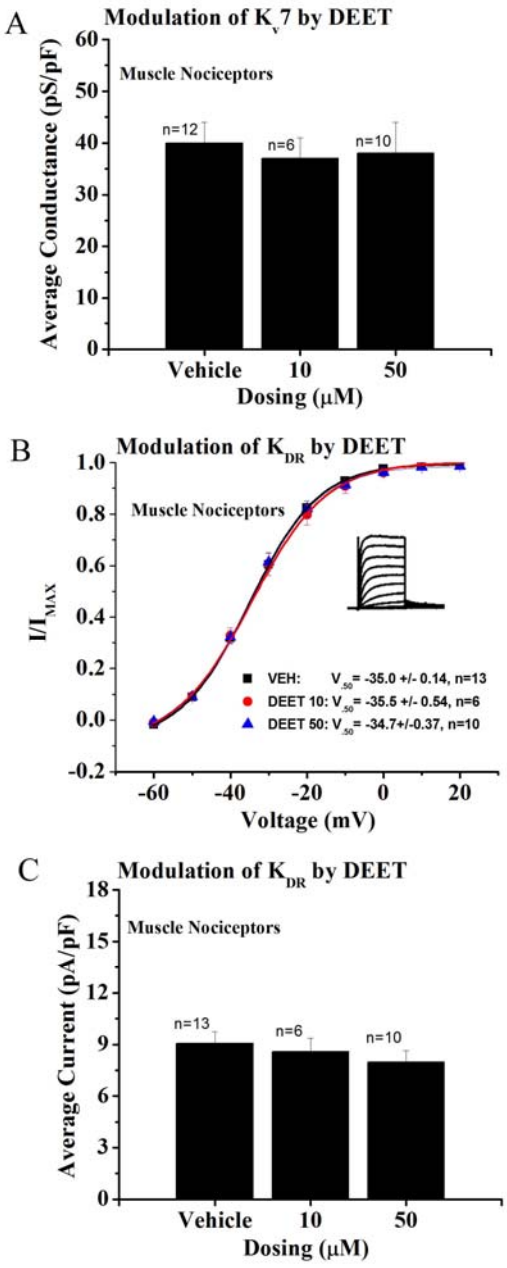

Figure 5. Voltage Activated K^+ Channels were Unaffected by Acute Exposure to DEET. A) The average conductance of muscle nociceptor K_v7 channels was not altered by DEET (10-50 μM); **B) and C)** Following a 12 minute exposure, there was no indication that either the voltage dependence or the average K_{DR} currents were modified by DEET (10-50 μM). Insert B: a representative family of K_{DR} current traces (-80 to 30 mV). The voltage activation curves shown were formed from the mean tail currents of all cells averaged at a given voltage. Statistical tests were performed on $V_{.50}$'s computed from individual curve fits. For K_v7, the average currents were determined as the mean linopirdine sensitive current from -40 to -70 mV. For K_{DR}, the average currents were determined as the mean tail current from -60 to 0 mV. Data was collected from 33 rats.

The Influence of DEET on $Na_v1.8$. Cells were plated as described above. Following identification of a neuron as a muscle or vascular nociceptor, $TTX_{insensitive}$ Na_v were isolated from other voltage activated currents using the Na_{iso} solution ($[Na^+]$ = 20 mM) and 500 nM TTX (described in 'Methods'). Time dependent changes to $Na_v1.8$, in the presence of DEET, were examined. Following a conditioning pulse to -70 mV a strongly depolarizing step to 0 mV (60 msec), evoked powerful inward currents (figure 6A insert). In the presence of vehicle (ETOH), a stable peak current was established over 6-10 evocations (pre-test series). Subsequently, DEET containing solutions were presented for 2 minutes by close superfusion (100 μM) and the protocol was then restarted and continued for 7 additional minutes in the continuous presence of DEET (post-test series; 15 second intervals; 28 total tests). In separate experiments, pre and post-test solutions contained only ETOH vehicle.

We compared the percentage change in leak corrected peak amplitudes of $Na_v1.8$ between ETOH baseline and DEET treated cases (post/pre). We found no evidence that a 9 minute presentation of 100 μM DEET could reduce the amplitude of $Na_v1.8$ in either muscle or vascular nociceptors (figure 6).

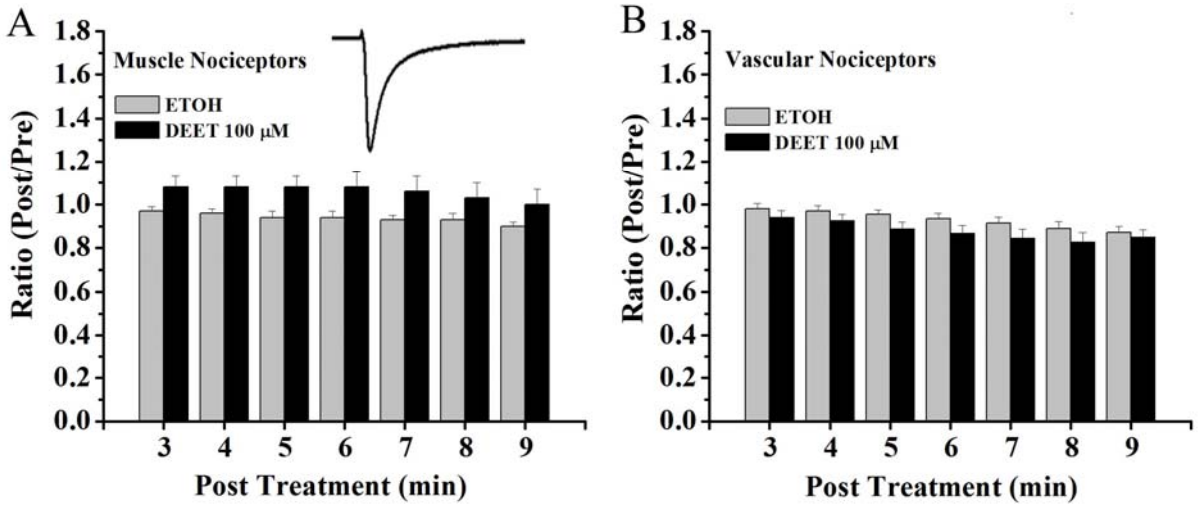

Figure 6. Time Dependent Modification of $Na_v1.8$ by DEET. A) The amplitude of muscle nociceptor $Na_v1.8$ was not changed by DEET (100 μM). A representative Nav1.8 current is inserted. **B)** Vascular nociceptor $Na_v1.8$ amplitude was not altered by DEET (100 μM). Baseline records were taken prior to DEET or ETOH exposure. The average of the last three pre-tests was used as a baseline score. DEET was pre-applied for 2 minutes prior to 7 minutes of continuous post-test recording (15 sec test intervals). This data was collected from 19 rats.

The Influence of DEET on $Na_v1.9$. Following an 8 week exposure to three GWI chemicals, the amplitude of $TTX_{insensitive}$, $Na_v1.9$ was significantly increased (Nutter et al., 2014). Although $Na_v1.9$ does not contribute to the formation of action potentials (Cummins et al., 1999; Dib-Hajj et al., 2002), it is an important contributor to nociceptor discharge properties (Fang et al., 2002; Jiang and Cooper, 2011). Due to its ultraslow kinetics and hyperpolarized voltage dependence, $Na_v1.9$ could mediate burst discharges via the formation of long duration 'plateau' potentials (Copel et al., 2009, Herzog et al., 2001; Maingret et al., 2008). If DEET influenced the amplitude of $Na_v1.9$ during exposure, it might amplify the post-exposure influence of GWI chemicals (permethrin, chlorpyrifos and PB) on the physiology of $Na_v1.9$. Accordingly, we examined whether an acute presentation of DEET would modify this unique $TTX_{insensitive}$ Na_v channel.

Muscle and vascular nociceptors were isolated as described above. A Na_{iso} solution ($[Na^+]$ = 70 mM; 500 nM TTX; see 'Methods') containing either DEET or vehicle (ETOH) was applied for 2 minutes. After this conditioning period, a family of voltage dependent currents were generated using stepped pulses from -80 to -20 mV (V_H= -120 mV, 5 mV steps, 300 ms duration). Leak corrections were performed on line and activation curves were constructed by fitting a Boltzmann function. Individual cases in which Boltzmann functions could not be fit were excluded from the analysis.

DEET exhibited some weak and inconsistent influences on $Na_v1.9$. Following application of DEET to muscle and vascular nociceptor $Na_v1.9$, there was some indication that high doses (>100 μM) might modulate the current. When the averaged peak amplitudes of the DEET treated currents were compared to vehicle treated cases, it appeared that trends favored a decrease in amplitude at doses exceeding 100 μM. All comparisons to vehicle treated cases were non-significant; however, a significant difference was observed between tests at 50 and 100 μM (figure

7C). In addition to the averaged current, we observed a significant ~-2 mV shift in the voltage dependence of muscle nociceptor $Na_v1.9$ ($V_{.50}$; 50 μM; figure 7A). However, we could not reproduce the voltage shift at 100 μM DEET. No effects of DEET were observed for voltage dependence of vascular nociceptor $Na_v1.9$ (figure 7A and B). As these shifts all occurred at what were clearly non-physiological doses, we did not pursue these trends any further.

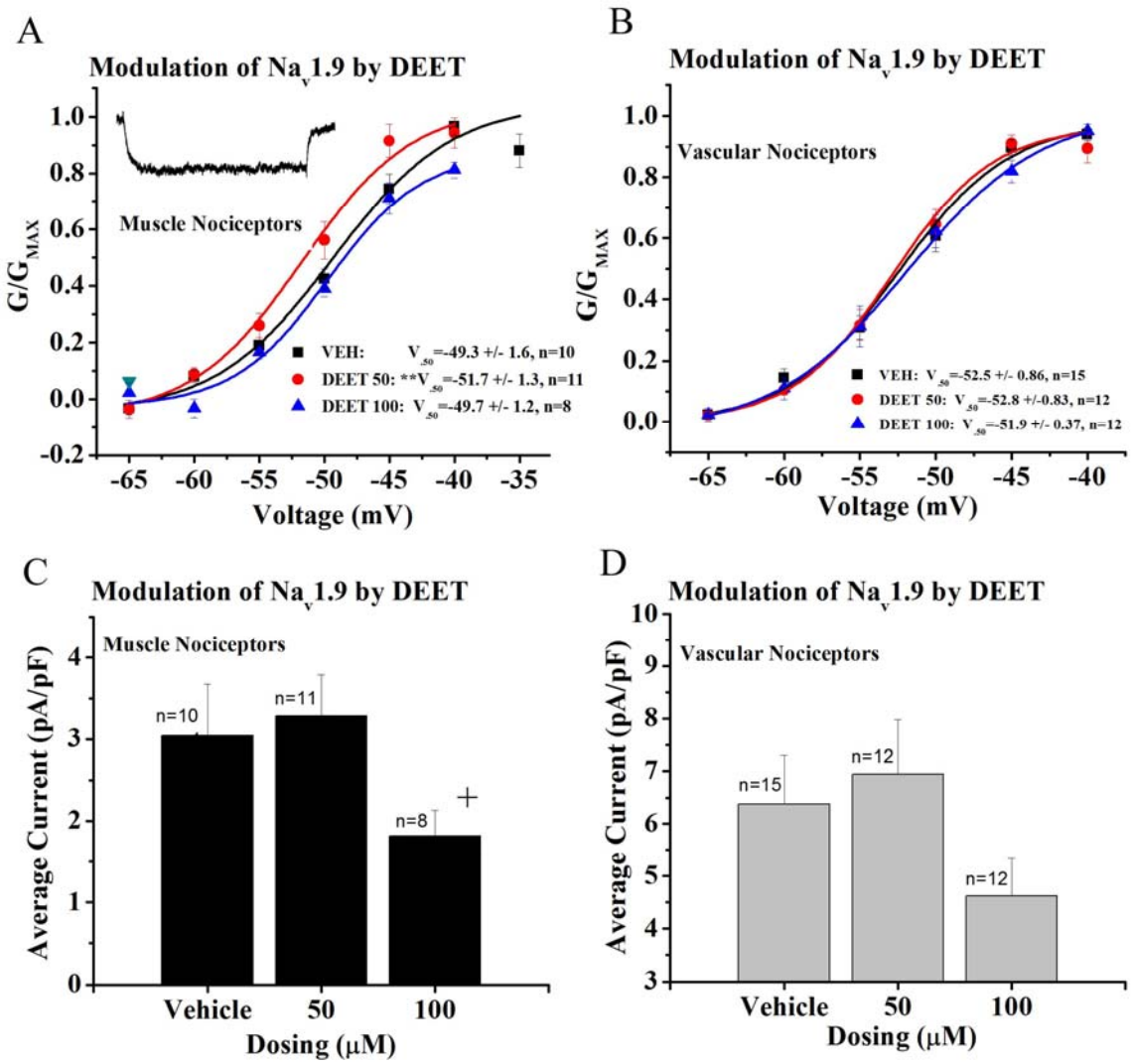

Figure 7. Voltage Activated, Na$_v$1.9, Channels were Weakly Modulated by Acute Exposure to DEET. A) The voltage dependent activation of muscle nociceptors was hyperpolarized at 50 but not at 100 μM DEET. A representative trace of a Na$_v$1.9 current (step to -50 mV; V_H=-120 mV) is included as an insert. **B)** The voltage dependent activation of vascular nociceptors was unaffected by DEET. **C)** Average currents of muscle nociceptors were unaffected by DEET (p<.13, Vehicle vs 100 μM; p<.05, 50 vs 100 μM); **D)** Vascular nociceptor average currents were unchanged (p<.17, Vehicle vs 100 μM; p<.08, 50 vs 100 μM). The voltage-activation curves were formed from the mean conductances of all cells averaged at a given voltage. Statistical tests were performed on V_{50} 's computed from individual curve fits. Average currents were determined as the mean, cell size normalized, current over the activation range (-65 to -40 mV). **significantly different from vehicle treated cases; + significantly different from 50 μM tests. Thirty-two rats contributed to these graphs.

Nociceptor Physiology in Rats Chronically Exposed to Combinations of DEET, Permethrin, PB and Chlorpyrifos.

We examined whether the, DEET augmented, 4 week exposure protocol altered muscle nociceptor physiology. Experiments were conducted on Group A, Group PB and Group C rats. Twelve weeks after exposures ended, DRGs were excised from rats for whole cell patch clamp studies. Recordings were obtained from muscle nociceptor (type 5) $Na_v1.9$ ion channels using solutions and protocols described in 'Methods'. Following preliminary characterization procedures, muscle nociceptor, $Na_v1.9$ currents were isolated from other voltage dependent currents. From a holding potential of -120 mV, currents were evoked in 5 mV steps (-80 to -20 mV). A voltage of half activation ($V_{.50}$) was determined following fit of the evoked currents to a Boltzmann equation.

Regardless of the presence or absence of PB, there was no indication that DEET augmented protocols altered the $V_{.50}$ of activation (figure 8A). In contrast, the average evoked current of muscle nociceptor $Na_v1.9$ was increased in both Group A and Group PB relative to vehicle exposed controls. Although distinct exposure protocols led to distinct behavioral outcomes (figures 1 and 4), there were no differences in $Na_v1.9$ amplitude in neurons harvested from Group A versus those from Group PB (figure 8B).

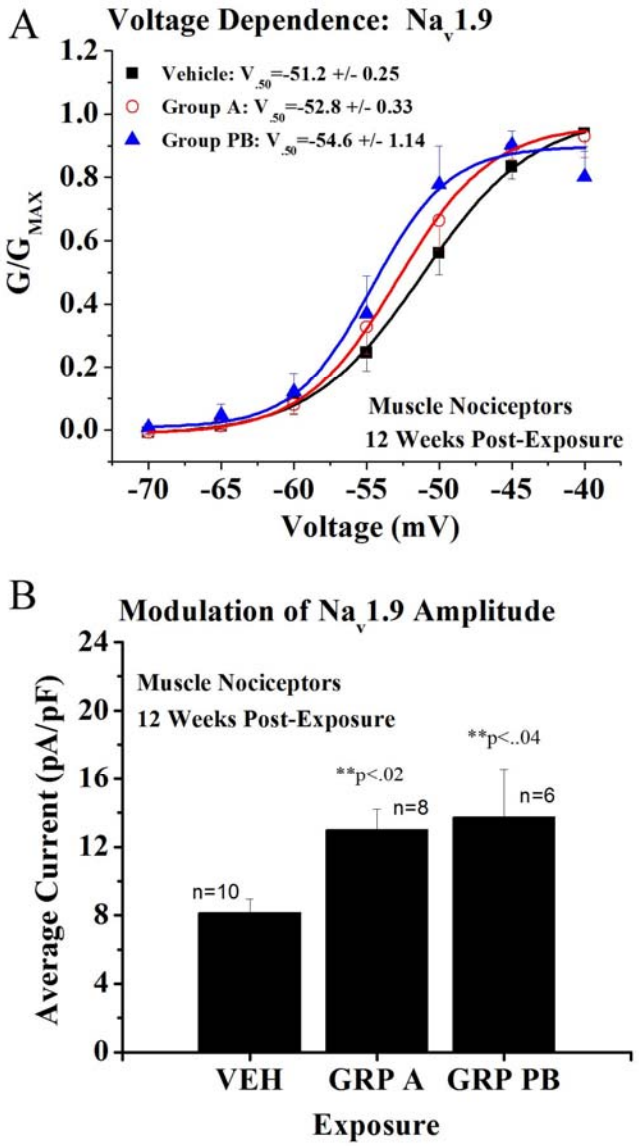

Figure 8. The Average Current of Muscle Nociceptor $Na_v1.9$ was Increased 12 Weeks Post-Exposure. A) The voltage dependent activation of $Na_v1.9$ was unchanged. The number of cells contributing to each curve are given in panel 'B'. **B)** Consistent with increased membrane excitability, the average current (-65 to -40 mV) was significantly increased in both Group A and Group PB muscle nociceptors. GRP A: PB, permethrin, chlorpyrifos and DEET; GRP PB: permethrin, chlorpyrifos and DEET; VEH: water, ETOH, corn oil, ETOH. To account for cell size variations, the average currents were normalized with respect to cell capacitance (pF). Thirteen rats contributed to these experiments. ** significantly different from vehicle exposed cases.

Discussion

We previously identified a protocol in which an 8 week, but not a 4 week, exposure to 3 GWI agents (chlorpyrifos, permethrin, PB) produced a pattern of behavior that could be interpreted as a delayed chronic pain syndrome that resembled a subset of symptoms that afflicted veterans following their return from the 1991 Persian Gulf War (Nutter et al., 2013; Nutter and Cooper, 2014; Nutter et al., 2015). A key factor leading to the development of those pain-like behaviors was a doubling of the anticholinesterase duty cycle from 7% (twice per month) and 50% (15 times per month) to 14% and 100% (chlorpyrifos, PB; respectively). Given that DEET usage has been statistically associated with the development of the GWI pain syndrome (syndrome 3; Haley and Kurt, 1997), we examined whether the addition of this repellant to our exposure protocol would promote the appearance of pain behaviors in our rat model. Our studies have now shown that DEET hastened the development and extended the persistence of pain-like behaviors that appeared following a 4 week exposure to chlorpyrifos, permethrin and PB. Relative to either vehicle or positive control groups, rat ambulatory behaviors were suppressed as early as 5-12 weeks post-exposure and remained suppressed out to the 17-24 week test periods. Consistent with the presence of a myalgia, these behavior shifts were accompanied by increased activity in muscle nociceptor $Na_v1.9$. Despite the powerful influence of DEET, the development and persistence of pain signs were shown to be mainly dependent, albeit in a complex fashion, on the anticholinesterase components of the exposure protocol.

Experiments in which chlorpyrifos or PB were eliminated from the exposure set gave clear indications of the importance of these anticholinesterases for the development and maintenance of pain behaviors. In the absence of CP, ambulation deficits were substantially reduced or prevented. The extent of reversals of post-exposure ambulation measures were even more complete in the absence of PB. This, despite the fact that removal of PB had much less impact on behavior patterns occurring during the exposure.

DEET Potentiates the Development of a Chronic Pain Condition

Despite the critical contribution of the anticholinesterases to the development of pain signs in our model, the fact that any ambulatory deficits appeared, following a 4 week exposure, seemed to be attributable to the inclusion of DEET in the exposure set. When the concentration level of DEET was halved from 50 to 25%, there was no indication that DEET potentiated or altered the pattern of pain signs. This finding is consistent with the observation that veterans that applied a 75%, but not a 25% concentration of a commercial DEET preparation were more likely to develop GW pain symptoms (Haley and Kurt, 1997). The means by which DEET amplified the impact of GW chemicals remains uncertain, but it is unlikely to be due to its weak anticholinesterase activity (Corbel et al., 2009; Wille et al., 2011; Swale et al., 2014).

We had reported previously that exposure to chlorpyrifos, permethrin and PB, at a low duty cycle, was insufficient to produce ambulatory and resting pain signs, but increased the activity of muscle nociceptor $Na_v1.9$ eight weeks following exposures (Nutter and Cooper, 2014). In the DEET augmented protocol, we now report a similar enhancement of $Na_v1.9$ activity that was manifested at the 12 week post-exposure interval. The production of 'plateau potentials' by $Na_v1.9$

could promote burst discharges from muscle nociceptor pools that would contribute to maintenance of a widespread myalgia (Copel et al., 2009, Herzog et al., 2001; Maingret et al., 2008; Nutter et al., 2015). It is possible that DEET intensified what had been a subclinical influence on $Na_v1.9$ to a level at which behavioral deficits were manifested. However, there is no specific evidence for that interpretation. It is simple to argue for a contribution of $Na_v1.9$ to the development and maintenance of pain signs, but it is difficult to see how dysfunctional $Na_v1.9$ currents could account, completely, for the observed patterns of ambulatory and/or resting pain.

The removal of PB from the exposure protocol shifted the pattern of behavioral signs, but there was no indication that this was reflected, in any way, by muscle nociceptor $Na_v1.9$. When PB was removed from the protocol, ambulation signs were substantially reduced and resting signs substantially increased. The amplitude of $Na_v1.9$ currents were not influenced by the presence or absence of PB in the exposure protocol. Finally, GW agents increased $Na_v1.9$ whether or not any pain signs were detected (Nutter and Cooper, 2014). We have reported that the physiology of other ion channels (K_{DR}, K_V7) can be altered by chronic exposure to GW agents. An examination of their properties might clarify the physiological influence of DEET and/or PB on pain system neurons (Nutter et al., 2015; Cooper et al., 2016).

While DEET clearly potentiated behavioral maladaptations, and may have potentiated shifts in muscle nociceptor physiology, we could not demonstrate any acute influences of DEET on $Na_v1.9$ activity. Nor did we observe acute influences, by DEET, on other ion channels that have been implicated in GWI agent-induced maladaptations (i.e., $Na_v1.8$, K_v7 and K_{DR}; Nutter and Cooper, 2014; Nutter et al., 2015; Cooper et al., 2016). The absence of effects of DEET on known and suspected GW agent maladapted ion channel proteins does not preclude a direct molecular

level interaction of DEET with deep tissue nociceptors through other pathways; nor does it rule out an indirect interaction through secondary pathways yet to be identified.

Chlorpyrifos is an AChE inhibitor, but one of its hepatic metabolites, chlorpyrifos-oxon is 1,000 fold more potent in that role (Huff and Corcoran, 1994). In a direct assessment of the hepatic interaction of these two GW agents, Usmani and colleagues reported that DEET increased the hepatic conversion of chlorpyrifos to its oxon form by a factor of 2.4 (Usmani et al., 2002). Potentially the amplification of pain signs, by DEET, occurs through its capacity to increase the peak levels of the more potent oxon form of CP (Abou-Donia et al., 1996; Abu-Qare and Abuo-Donia, 2008). Moreover, when PB was co-administered with DEET, it slowed hepatic DEET metabolism, and as a result could further enhance the DEET potentiated conversion of chlorpyrifos to chlorpyrifos-oxon (Abu-Qare and Abu-Donia, 2008; see also Chaney et al., 2000). Therefore, the amplification of pain signs by addition of DEET, in an otherwise ineffective protocol, could simply be due to an increase in the effective peak concentration of chlorpyrifos-oxon. Just as increasing the duty cycle of the anticholinesterases potentiated pain signs with an 8 week exposure (Nutter et al., 2015), increasing the functional concentration of chlorpyrifos-oxon, during a 4 week exposure, could have potentiated and prolonged ambulatory deficits.

It is well known that single high dose exposures to certain organophosphate anticholinesterases have been linked to the development of a progressive motor and sensory neurodegenerative disorder known as OPIDN/OPIDP (organosphophate induced delayed neuropathy/polyneuropathy; Johnson, 1975; Vale and Lotti, 2015). One to three weeks after a single exposure, subjects develop a variety of symptoms that include progressive ataxia, paresthesias, and muscle pain. These are accompanied by a peripheral axonopathy characterized by slowed action potential conduction and evidence of Wallerian degeneration (Lotti and Moretto,

2005). Most animal studies have been conducted on 'susceptible' species (chickens and mice). Severe inhibition of NTE (neuropathy target esterase) and induction of intracellular, phosphorylation pathways are key events leading to the development of the axonopathy associated with OPIDN (Abou-Donia and Lapadula, 1990; Pope et al., 2005; Choudhary et al., 2006; Emerick et al., 2012). However, rats are considered to be highly resistant to OPIDN (but see Padilla and Veronesi, 1988; Moretto et al., 1992; Choudhary et al., 2001), and there is little evidence that chickens, mice or rats, develop classic signs of OPIDN following single or multiple exposures to either chlorpyrifos or PB (Richardson, 1995; Lotti, 2002a; Wilson et al., 2002; Kropp and Richardson, 2003; Wang et al., 2014). Although sequential exposure to some organophosphates (including chlorpyrifos) with certain 'non-neuropathic' NTE inhibitors potentiates organophosphate inhibition of NTE and ultimately leads to the development of OPIDN (Pope and Padilla, 1990; Pope et al., 1993; Lotti et al., 1991; Lotti, 2002b), we cannot point to any specific evidence linking PB, permethrin or DEET to promotion of a chlorpyrifos dependent OPIDN.

Complex Influences of Chlorpyrifos and PB on Pain Signs

The evidence that linked the anticholinesterases to the development of ambulation deficits was relatively straightforward. The contributions of CP and PB were internally consistent on these measures: exclusion of either CP or PB blocked the development of ambulation pain-signs and significantly shifted ambulation scores that accompanied 4 agent exposures, toward normal levels. The exclusion of PB was more definitive in this regard, as its presence was required for the development of both movement distance and rate impairments (weeks 5-12). However, both the anticholinesterases were required for the persistence of ambulation deficits out to 20 weeks

post-exposure. Unexpectedly, we observed that the development of rest duration pain signs diverged from those of ambulation. Resting increased during the post-exposure phase *only* in the absence of PB. Moreover, resting pain signs were further increased relative to groups receiving all four chemicals. The divergent influence of the two anticholinesterases on rat activity measures must be considered, not only with respect to their interactive anticholinesterase activity, but also in the broader context of their non-cholinesterase interactions with the nervous system.

PB, a reversible inhibitor of AChE, was approved for use in ODS due to its capacity to act as a prophylactic, and when combined with antidotes (2-PAM; atropine) to reduce the lethality of highly potent and irreversible anticholinesterase nerve agents such as Soman (Maxwell et al., 1988; von Bredow et al., 1991; Adle et al., 1992; Koplovitz and Stewart, 1994; Kassa and Fusek, 1998; Weinbroum, 2004; Newmark, 2005; Weissman and Raveh, 2011). Chlorpyrifos-oxon is an irreversible anticholinesterase whose anticholinesterase activity is significantly reduced by pre-treatment with PB (Henderson et al., 2012). When PB was left out of our dosing protocol, the loss of this prophylactic action might be manifested in the development of resting pain signs. In partial support of this, we have shown that increasing the duration of exposure to CP to 8 weeks, also increases resting scores in weeks 9-12 (Nutter et al., 2015; Cooper et al., 2016).

While PB has a demonstrated capacity to oppose the anticholinesterase effects of chlorpyrifos-oxon (Henderson et al., 2012), it has no capacity to oppose or prevent the extra-anticholinesterase effects of chlorpyrifos-oxon. The latter are considerable and well documented. Independent of any inhibition of AChE, chlopryrifos-oxon, as well as some other organophosphates used in the Gulf War (i.e., malathion/maloxon) can directly bind to, activate, and cause internalization of muscarinic receptors (Ward et al., 1993; Huff et al., 1994; Ward and Mundy, 1996; Bomser and Casida, 2001; Olivier et al., 2001; Howard and Pope, 2002; Liu et al.,

2002; Zou et al., 2006; Mirajkar and Pope, 2008; Udarbe et al., 2008; see also Smulders et al., 2004). Chlorpyrifos-oxon also modulates the activity of a variety of G-protein coupled protein kinases and receptor protein kinases (Huff et al., 1995; Huff et al., 2001; Bomser and Casida, 2000; Bomser et al., 2002; Zhang et al., 2002; Torres-Altoro et al., 2011; Suriyo et al., 2015). Repeated exposure to chlorpyrifos ultimately alters the expression of muscarinic receptors in the CNS (Nostrandt et al., 1997; Liu et al., 1999; Huff et al., 2001; Abou-Donia et al., 2003; Zhang et al., 2002; Padilla et al., 2005; Pung et al., 2006; Proskocil et al., 2010) and modifies the functional consequences of mAChR activation in PNS nociceptors (Nutter and Cooper, 2015; Cooper et al., 2016).

How these multiple cholinesterase and non-cholinesterase actions contribute to the development of specific pain signs cannot be readily resolved in this report. Our examination of muscle nociceptor $Na_v1.9$ did not reveal any differential influences attributable to the presence or absence of PB in the exposure protocol (see above). Soldiers suffering from GWI pain describe highly diverse symptom patterns (i.e., muscle pain, back pain, joint pain, abdominal pain, headache) that suggest pathology in multiple, functionally distinct, nociceptor pools that innervate these diverse tissues (Blanchard et al., 2006; Stimpson et al., 2006; Thomas et al., 2006; Haley et al., 2013). In the absence of PB, additional nociceptive pools, under the full influence of both the anticholinesterase and extra-cholinesterase actions of CP, could suffer significant maladaptations that are manifested as distinct forms of pain.

Summary and Conclusions

DEET substantially potentiated and prolonged the pain signs that developed after a 4 week exposure to GW agents. The pattern of pain signs associated with a DEET augmented exposure set diverged from those observed after an 8 week exposure to the same GW agents in the absence

of DEET (Nutter et al., 2015; Cooper et al., 2016). While we were unable to identify a specific linkage between nociceptor ion channel physiology and acute exposure to DEET, such interactions may yet be found. At present, it is more likely that DEET indirectly amplifies the physiological impact of chlorpyrifos on a variety of molecular targets.

Soldiers deployed to the Persian Gulf were potentially exposed to a large variety of insecticides, repellants, nerve agents, adjuvants, depleted uranium, and other toxins (Binns et al., 2008; RAC, 2014). A high percentage developed symptoms while still in theater (~25%; Kroenke et al., 1998), but most developed a wide variety of pain symptoms that were delayed in onset and worsened over time (Hotopf et al., 2003). The variations of the symptoms, as well as the timing of their onset, could represent different exposure patterns (and degrees of exposures) and how they ultimately interacted with the genetic makeup of each individual. Acknowledging that, it is likely that there were common risk factors that set into motion a definable set of maladaptations that resulted in the symptoms of GWI. Most of our research points to the fundamental role of the anticholinesterases, CP and PB, as primary risk factors for pain. Although it is not clear that it was specifically their anticholinesterase activity that posed the greatest risk, the manifestations of pain-like behaviors appeared after doubling the exposure duty cycle of PB and CP (Nutter et al., 2015; Cooper et al., 2016). Adding DEET to the exposure set accelerated the development, altered the pattern and prolonged the persistence of pain-like behaviors that were ultimately dependent upon the presence of chlorpyrifos and/or pyridostigmine bromide. The impact of DEET augmentation on muscle nociceptor $Na_V1.9$ physiology might account for some of the resultant behavioral signs, but $Na_V1.9$ status could not account for the differential influences of PB on these signs.

PB was prescribed to soldiers to protect them from nerve agents such as Soman or Tabun (Gordon et al., 1978; Gall, 1981; Ray et al., 1991; Adler, et al., 1992; Kassa and Vachek, 2002; Kassa and Krejeova, 2003; Maselli et al., 2011; but see Shiloff and Clement, 1986). The full benefit of PB pre-treatment required timely administration of antidotes, such as 2-PAM and atropine (Maxwell, et al., 1988; von Bredow et al., 1991; Adler, et al., 1992; Koplovitz and Stewart, 1994; Kassa and Fusek, 1998; Kassa and Vachek, 2002; Layish et al., 2005). Ironically, Soman was never encountered in the Persian Gulf; and while Sarin nerve agent was encountered, PB had not been shown to be a useful prophylactic against Sarin (Koplovitz et al., 1992; Worek and Szinicz, 1995; Wilson et al., 2002; but see Tuovinen et al., 1999). As this could not be known beforehand, measures were taken that were believed to offer the best margins of safety for the warfighters. Probably half of the soldiers deployed to the Persian Gulf self-administered PB, without antidote, for several weeks. The antidotes were not to be taken unless there was an indication that a nerve gas attack was imminent or in progress (Binns et al., 2008). Accordingly, soldiers took PB routinely in anticipation of attacks that rarely, if ever, materialized and for which its prophylactic action was documented to be of little use. As a result, they may have been self-administering an agent that accentuated the toxic effects of insecticides and repellants through an hepatic overload (Abou-Donia et al., 1996). Nevertheless, as the present data indicates, routine administration of PB did afford a degree of protection against the physiological impact of some of the anticholinesterase insecticides to which the soldiers were overexposed, and whose toxicity was amplified by what was thought to be a harmless repellant (DEET). Yet, PB could not protect them from, and may have actually amplified the actions of, the oxon metabolites of the organophosphates that asserted their deleterious actions through pathways that were independent

of anticholinesterase activity but had the capacity to derange important components of the nervous system.

Acknowledgements

Studies were funded by DoD W81XWH-14-GWIRP-IIRA, GW120066 to BC and RDJ and by the University of Florida Research Foundation (BC).

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